

MED
T113
+Y12
7251

YALE UNIVERSITY LIBRARY



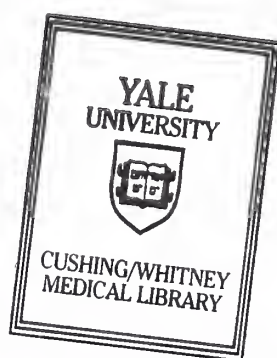
39002079257110

The Ventilatory Response to Normoxic Hypercapnia in
Male Pde1 Knockout Mice and the Influence of
Female Sex Hormones

Simon Koderick Alfred Host

YALE UNIVERSITY

2006




Permission to photocopy or microfilm processing of this thesis for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.



Signature of Author

4/10/06

Date



Digitized by the Internet Archive
in 2017 with funding from
Arcadia Fund

<https://archive.org/details/ventilatoryrespo00best>

The Ventilatory Response to Normoxic Hypercapnia in Male Pet-1 Knockout
Mice and the Influence of Female Sex Hormones

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Simon Roderick Alfred Best

2006

YALE MEDICAL LIBRARY

AUG 12 2006

T113
+ Y12
7251

Abstract

THE VENTILATORY RESPONSE TO NORMOXIC HYPERCAPNIA IN MALE PET-1 KNOCKOUT MICE AND THE INFLUENCE OF FEMALE SEX HORMONES.

Simon R. A. Best and Matthew R. Hodges (Sponsored by George B. Richerson).

Department of Neurology, Yale University, School of Medicine, New Haven, CT.

Purpose: To determine if the deficiency in the hypercapnic ventilatory response as a result of 70% loss of serotonergic neurons in male Pet-1 knockout mice and the sparing of the female Pet-1 knockout mice can be accounted for and corrected by supplementation with estrogen and progesterone.

Methods: Using whole-body flow-through plethysmography, 5 male Pet-1 $-/-$ mice and 5 wild type mice were exposed to room air, 3%, 5%, 7%, and 10% CO₂ and V_E measured. All mice were then treated with oral 17 β -estradiol (2500 nM) for 48 hours followed by a SC injection of progesterone (2 mg/kg). One hour after injection all plethysmography studies were repeated.

Results: Before treatment Pet-1 knockout mice showed a trend towards decreased V_E at room air, 3%, and 7% CO₂, with statistical significance at 5% CO₂ ($p < 0.05$), and an identical V_E only at 10% CO₂. Absolute changes in V_E after treatment were insignificant; the differences in V_E between genotypes achieved significance at control, 3%, and 5% CO₂ ($p < 0.05$) while now V_E at 7% and 10% CO₂ exposures were identical ($p < 0.05$).

Conclusion: The partial recovery of the male Pet-1 knockout phenotype provides evidence that the remaining serotonergic neurons can recover some chemoreceptor function under the influence of female sex hormones, which in turn may partially explain the female Pet-1 knockout phenotype.

Thank you to all the members of the Richerson lab for their assistance, advice, and knowledge throughout this process. Specific thanks to John Sayward for his computer programming skills and to Matthew Hodges for his role in the plethysmography design, troubleshooting, and experimental assistance.

To Dr. George Richerson, whose tireless, rigorous, and ethical pursuit of the truth represents the best of the human intellectual spirit and is an inspiration to all who work in his lab.

Table of Contents

1) <i>Introduction</i>	5
2) <i>Statement of Purpose</i>	15
3) <i>Methods</i>	16
4) <i>Results</i>	23
5) <i>Discussion</i>	34
6) <i>References</i>	46

Introduction

As a neurotransmitter and a neural system, serotonin has been implicated in a wide range of functions and disorders. These include the regulation of sleep, aggression, anxiety, and clinical disorders such as depression, Obsessive Compulsive Disorder and SIDS. While there is much clinical, pharmaceutical, and physiologic evidence to support serotonin's role in these various functions, little attention has been paid to a unifying theory of the serotonin system that could account for these diverse roles. Given its small, centrally located nuclei and its extensive connections throughout the CNS it is not unreasonable to assume that some primary function could provide the key to its multifaceted phenotype.

Richerson has recently proposed central chemoreception as such a unifying function for the serotonin system that takes into account some of primary properties of serotonin neurons as well as provides an explanation for its role in these diverse disorders (1).

Chemoreception is the “detection” component of the fundamental homeostatic mechanism that is necessary to maintain a body pH between tight physiologic boundaries. The primary determinant of pH in humans is carbon dioxide, through its stoichiometric and enzymatic conversion to bicarbonate and a proton ($\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}^+ + \text{HCO}_3^-$). Therefore, candidate chemoreceptors must be able to respond to changes in pH by initiating appropriate physiologic defense mechanisms. In the case of pH, the most important acute response is the maintenance of P_{CO_2} by the control of lung ventilation.

The first central chemoreceptors were located by focal acidosis of the cat medulla, and an increase in ventilation was in seen on the ventrolateral surface (VLMS) (2). This

is the location for the nuclei of multiple types of neurotransmitters, including serotonin, whose medullary groups also include the raphe pallidus, raphe magnus, and raphe obscurus (3). These serotonergic projections influence cardiovascular control, autonomic output, motor control, and pain processing (4-6). They also have a well-described effect on breathing. Respiratory nuclei including the nucleus ambiguus, nucleus tractus solitarius, phrenic motor nuclei, hypoglossal nuclei, and the preBotzinger complex contain serotonergic terminals (7-12). Exogenous application of serotonin analogues stimulates respiration in rats and cats (13, 14). This effect is even more pronounced after general anesthetic, where respiratory depression or apneusis can be reversed with serotonin agonists (12, 15). Electrical stimulation of the raphe pallidus or glutamatergic stimulation of the medullary raphe both increase phrenic nerve output (16, 17). As would be expected, selective ablation of serotonergic nuclei leads to hypoventilation (18). In sum, the primary respiratory effect of serotonin is a stimulation of breathing, thus, if it could be shown that these neurons are chemosensitive, the hypothesis that serotonin neurons in the VLMS are responsible for pH homeostasis would be an attractive one.

The lines of evidence that indicate serotonin neurons have the necessary “detection” properties required for central chemoreception can be broken down into broad categories - (1) in vitro work with single cell culture, (2) specialized anatomic considerations, (3) in vivo studies with focal inhibition or destruction of serotonin neurons, and most recently (4) genetic manipulation of the serotonin system.

In vitro

The first clue that serotonergic neurons were involved in chemoreception came from rat brain slices, where a subset of neurons in the medullary raphe were found to be highly stimulated by changes in P_{CO_2} from 5% to 9% and inhibited by change to a P_{CO_2} of 3% (19). Another group of neurons showed an opposite response – they were inhibited by acidosis and stimulated by alkalosis. Of these subsets, all carbon-dioxide stimulated neurons were serotonergic, and none of the carbon-dioxide inhibited were. In cell culture experiments it was determined that 75-90% of all serotonin neurons in the medullary raphe respond to CO_2 with an average response of 300% increase in firing frequency (20-22). The channel responsible for this sensitivity appears to be a slow afterhyperpolarization calcium-activated cation current (23).

Anatomic Specialization

A striking structure-function relation exists for the serotonergic neurons in the brainstem. Confocal studies show that serotonergic neurons wrap closely along side the main arteries of the brain stem – the basilar artery and its penetrating branches – with dendrites that enwrap the vessels and come within half of a micrometer from the arterial blood (24). Teleologically, this is the ideal location for central chemoreceptors, as the basilar artery contains blood with a P_{CO_2} directly from the lung, reflecting gas exchange before it is altered by brain metabolism. This anatomical specialization has not been described in other putative chemoreceptors.

In vivo

In whole animal studies there is also evidence that serotonergic neurons contribute to the hypercapnic response. After hypercapnic exposure, *C-fos* staining increases in CO₂ activated neurons – these neurons in the medullary raphe are immunoreactive for 5-HT and tryptophan hydroxylase (25, 26). In awake, normally behaving cats, a subset of serotonergic neurons increases their firing rate in response to exposure to CO₂ (27). Two experiments inducing focal acidosis in the medullary raphe – by application of acetazolamide or by microdialysis of high CO₂ solution – both showed an increase in ventilation (28, 29). Similar findings have been achieved in goats, where focal acidosis in the medulla increases respiratory output (30, 31).

Experiments to inhibit or destroy serotonin neurons in the medullary raphe have also shown the expected results. When neonatal rats are treated with 5,7-DHT, which destroys serotonin neurons, in adulthood they have elevated P_{CO2} levels at baseline and a decreased ventilatory response to CO₂ (18). Focal lesions of the serotonin system in adult rats with 8-OH-DPAT or antibody conjugated to the toxin saporin directed at the 5-HT transporter cause a decrease in the ventilatory response to carbon dioxide (32, 33).

Genetic

Most recently, the ability to manipulate the serotonin system through genetic knockouts eliminates the need for invasive or chemical manipulations (34). Due to the high interest in the serotonin system for the study of behavioral and psychiatric systems, many variant knockout mice have been developed. One knockout generated is deficient in Pet-1, an ETS domain transcription factor critical for the normal development of

serotonin neurons. Mice deficient in Pet-1 lack 70% of their serotonin neurons in all serotonergic nuclei, with no detectable deficiencies in other neurotransmitter systems or brain architecture. The remaining serotonin neurons have dramatically reduced TPH and SERT mRNA expression. These deficiencies result in only 10-15% of wild-type serotonergic fibers and quantitative levels of serotonin throughout the brain.

Behaviorally these mice are more aggressive than their wild-type kindred, but have normal development, motor function and learning, reproduction, and life expectancy in adulthood, although 30% of them die in the first week of life from unknown causes (35). If the serotonin system was involved in chemoreception, Pet-1 knockout mice might be expected to have a deficient response to hypercapnia if peripheral chemoreceptors and the remaining serotonin neurons were unable to compensate sufficiently.

Indeed, recent studies with normoxic hypercapnic challenges have shown that Pet-1 knockout mice do not have a normal response [Fig 1 (a)] (36). This deficit appears to be mild, but when the results are sorted by the sex of the mice, a striking picture emerges, where the male mice have a clearly deficient response [Figure 1 (b)] and the female response is essentially normal [Figure 1 (c)]. This intriguing result suggests that some factor or factors are responsible for protecting the female mice from the knockout phenotype.

Ventilation of Pet-1 Knockout Mice

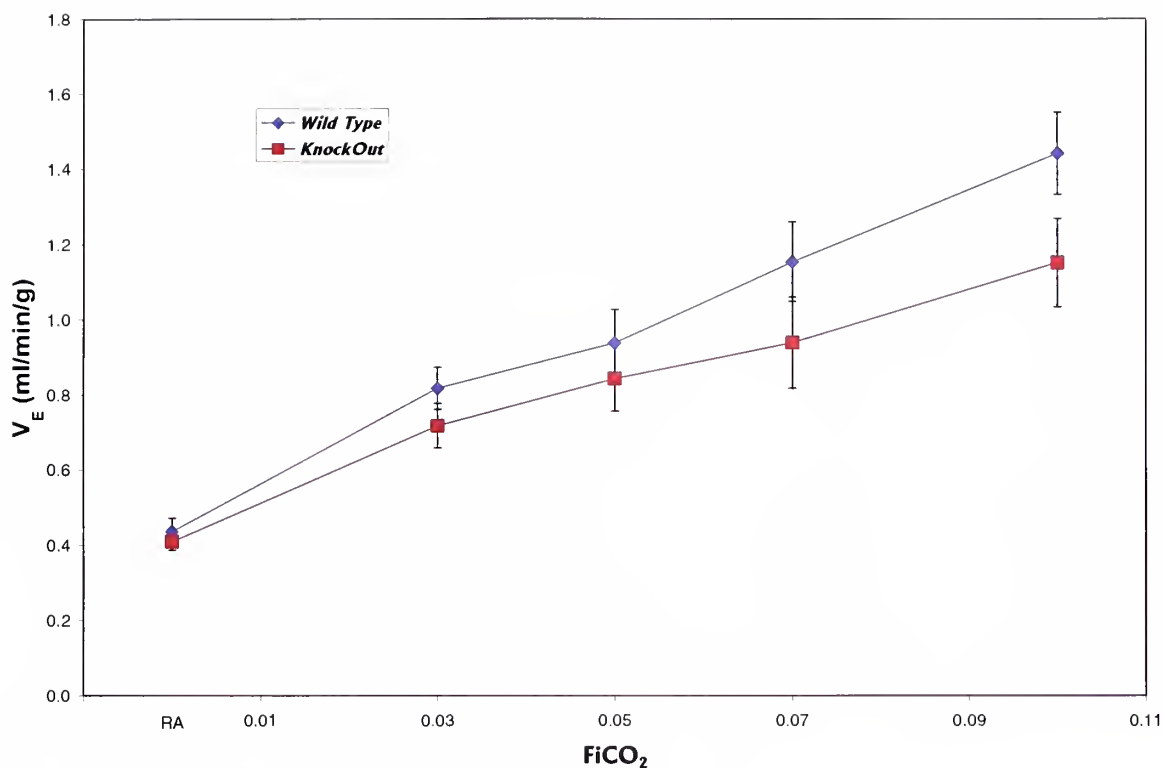


Figure 1 (a)

The most obvious source of sex differences is the influence of the female sex hormones estrogen and progesterone, especially since there is a well documented stimulatory effect of female sex hormones upon breathing, primarily progesterone, in humans and other species (37, 38).

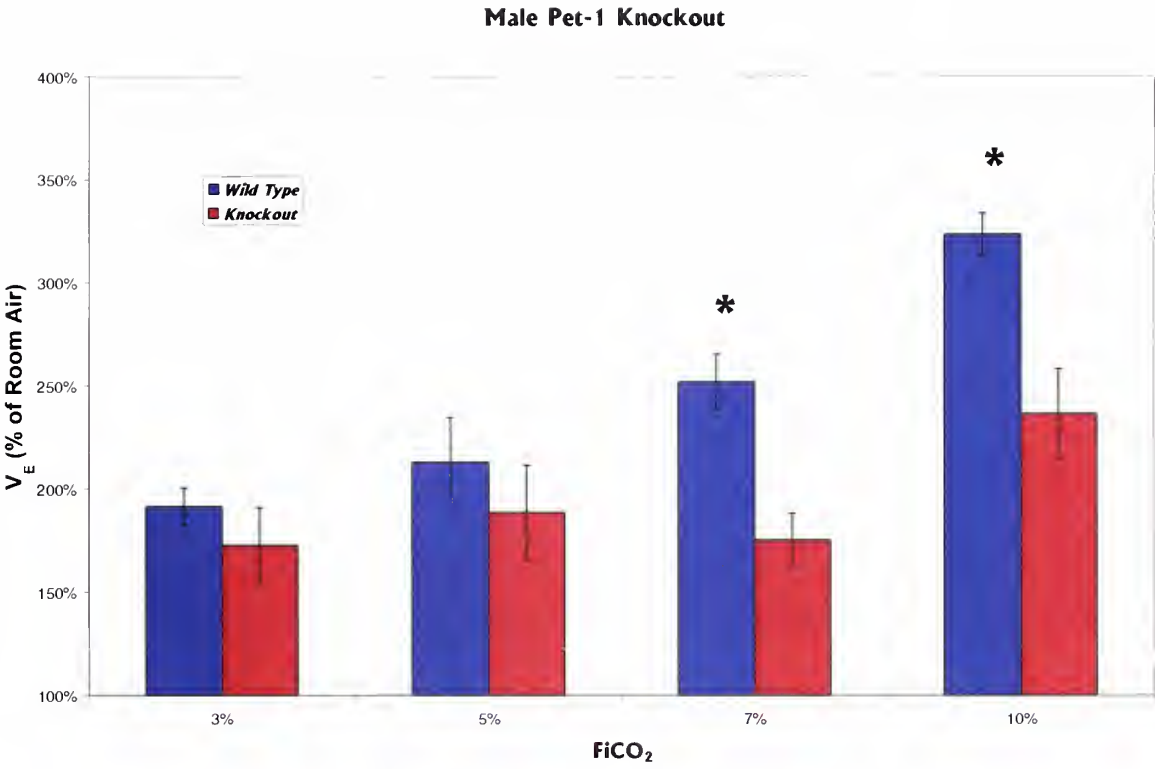


Figure 1 (b)

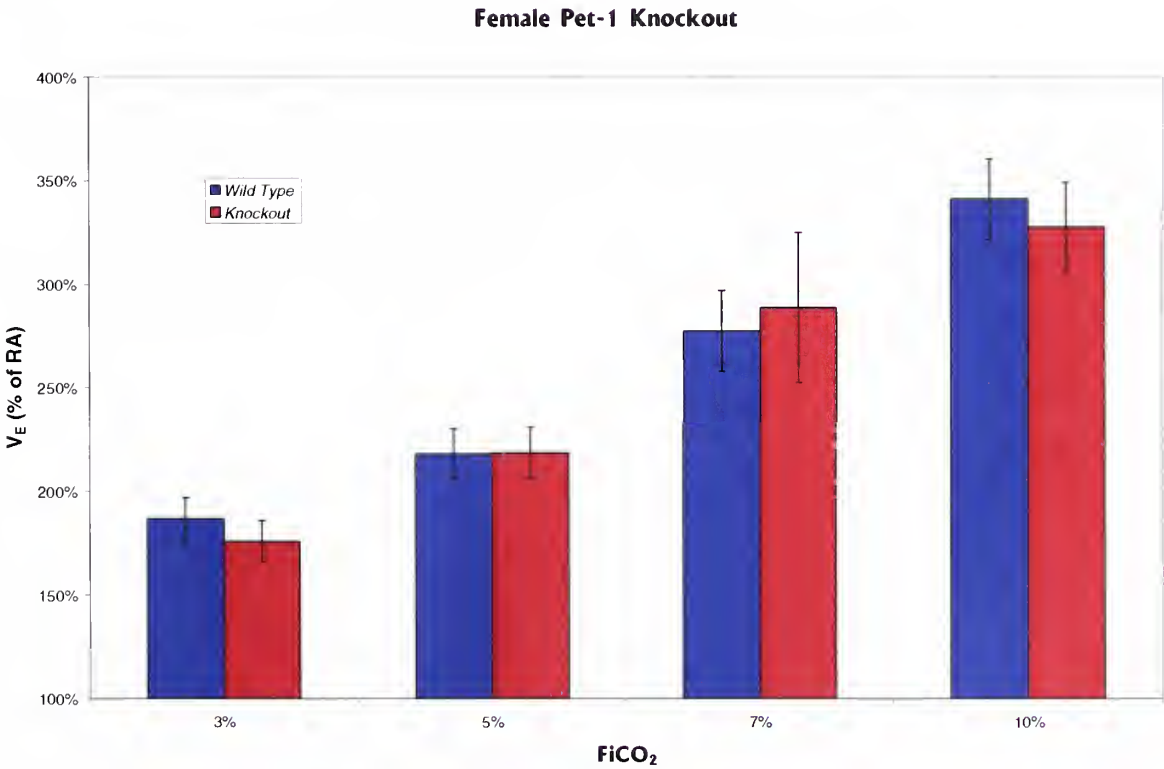


Figure 1 (c)

Sex Hormones and Respiration

Very early evidence for the effect of sex hormones on respiration was the observation that pregnant women have decreased alveolar and arterial P_{CO_2} (39, 40). The same effect was also seen during the luteal of the menstrual cycle (41) and these cyclic fluctuations disappeared after menopause (42). These observations were then correlated with progesterone (43), and further in pregnant women as the log of serum progesterone (44).

Progesterone was then shown to be a respiratory stimulant in normal, healthy men (45) in multiple physiologic states including exercise and sleep (46). Clearly men do not have the progesterone influence of pregnancy, but the one instance of a hyper progesterone and estrogen state in males is cirrhosis of the liver. Cirrhotics have long been known to have a baseline respiratory alkalosis, and when a group of patients with liver disease were divided into those with severe or mild hyperventilation those with greater hyperventilation had higher serum levels of estrogen and progesterone (47). It is interesting to note that the levels of progesterone that apparently stimulated breathing in cirrhotics were two orders of magnitude less than that in pregnant woman, which may reflect different sensitivities to the effect of sex hormones.

These hormones may have an effect on common respiratory diseases in humans, specifically Obstructive Sleep Apnea (OSA), which has a strong male predominance (48). Women are protected until menopause (49) - 11% of premenopausal women suffered from nocturnal desaturation, hypopneas or apneas during sleep in one study, while 60% of postmenopausal women had the same symptoms (50). There is also evidence that women taking hormone replacement therapies were protected (51).

Synthetic progestins (most commonly Medroxyprogesterone [MPA]) were therefore used as a treatment for OSA, and some studies showed improvements in blood gas levels, sleepiness, and apneic events in males (52-54). Since estrogen increases progesterone receptors (55) in females a combination hormone replacement therapy incorporating estrogen reduced sleep associated breathing disorders (56-58).

The ventilatory effects of estrogen and progesterone have also been duplicated in other animal systems. Guinea pigs (59), Jersey cows (60), rabbits (61), and goats (62) hyperventilate when they are pregnant. However, chronic progesterone to male rabbits and goats did not increase ventilation (63). Female rats do not have a ventilatory response to MPA alone, although when MPA is combined with estrogen the number of progesterone receptors increases and ventilation increases (64). This finding was duplicated in male rats, where only the combination of estrogen and a progesterone analog had an effect on arterial blood gas parameters (65). Work in cats showed the same requirement for estrogen and progesterone and further refined the model by blocking the response with an estrogen receptor antagonist, progesterone receptor antagonist, and inhibitors of protein and RNA synthesis (66). Therefore it was concluded that the ventilatory response is dependent upon both receptors as well as gene expression, consistent with the known mechanism of steroid receptor activation.

There are a few mechanisms possible for the ventilatory changes seen as a result of female sex hormones. The most attractive for the purposes of the current study is that estrogen and progesterone alter the hypercapnic ventilatory response (HCVR) ie) they influence central chemoreception. In men, administration of progesterone stimulated ventilation and also increased the HCVR versus placebo without changing the hypoxic

ventilatory response (45). In women, the change in ventilation in the luteal phase of the cycle was shown to be due to a difference in the carbon dioxide sensitivity (67). In post-menopausal women administration of estrogen and progesterone combined with mild exercise (to increase metabolic rate) caused an increase in ventilation that mimicked the effects of pregnancy. The authors showed that both progesterone alone and the combination of estrogen and progesterone caused a significant change in the HCVR (68). Finally, in pregnant women themselves, a challenge with hyperoxic hypercapnia showed a significant difference in both the HCVR and the threshold at which this response was activated (69).

Certainly other mechanisms besides a change in the HCVR could be involved. Both male and female rats express $ER\alpha$, $ER\beta$, and androgen receptors in the hypoglossal and phrenic motoneurons and nuclei, although no progesterone binding was seen (70). Sex hormones could be functioning as amplifiers of the normal HCVR simply by driving stronger motor output. They could also change the basic respiratory rhythm, however, there is no evidence that respiratory pattern generation generated by the pre-Bötzinger complex is altered by sex hormones (38).

Female sex hormones clearly have an influence on respiratory parameters across a wide variety of species, and perhaps even a specific effect on the hypercapnic ventilatory response. Therefore we hypothesized that supplementation of the male Pet-1 knock-out mice with physiologic levels of estrogen and progesterone would partially or completely restore the deficiency in their normoxic hypercapnic ventilatory response.

Statement of Purpose

The specific aims of this study were therefore:

- 1) To confirm the deficient ventilatory response of male Pet-1 knockout mice in response to normoxic hypercapnic challenges through whole-body plethysmography
- 2) Supplement the same male Pet-1 knockout mice with estrogen and progesterone and quantify the change, if any, in their hypercapnic ventilatory response as a result of hormone treatment

in order to:

Prove or disprove the hypothesis that the female sex hormones estrogen and progesterone are responsible for the sex differences in the hypercapnic ventilatory response of male and female Pet-1 knockout mice.

Methods

The protocol followed for these experiments was based upon a modified experimental design for previously published data (36) and approved for use by the Yale Animal Care and Use Committee.

10 male PET-1 mice (5 PET-1 $-/-$; 5 PET-1 $+/+$) generated from a breeder colony generously donated by the Deneris lab at Case Western were utilized for these experiments. All animals were kept in the Yale Animal Care Facilities and provided standard care – 12 hour diurnal cycles with freely available food and water.

Plethysmography

All plethysmography experiments were carried out in the following manner with the investigator blind to the mouse genotype. The mouse to be studied was weighed prior to experimentation and the ambient barometric pressure recorded. The mouse was then placed inside the airtight plethysmography chamber [Figure 2 (a)] which was connected to a gas manifold, a vacuum with a flow regulator, and a pressure transducer. When sealed, the plethysmography chamber and pressure transducer records a change in pressure that varies directly with the animal's breathing as the inhaled gas is warmed and humidified in the lungs (71). A custom designed Matlab 7.0.2 program then converted the output voltage of the transducer into a linear trace of the pressure in the chamber, while simultaneously recording the voltage at a sampling rate of 100 Hz. The pressure in the box could therefore be monitored to insure an accurate ventilatory signal and prevent a buildup of negative or positive pressure in the closed system. An extra chamber with a

needle valve connected to the main plethysmography chamber provided an additional pressure release point while buffering the chamber against sudden changes in atmospheric pressure (such as a door opening in the laboratory). This design is consistent with standard procedures for plethysmography experiments.

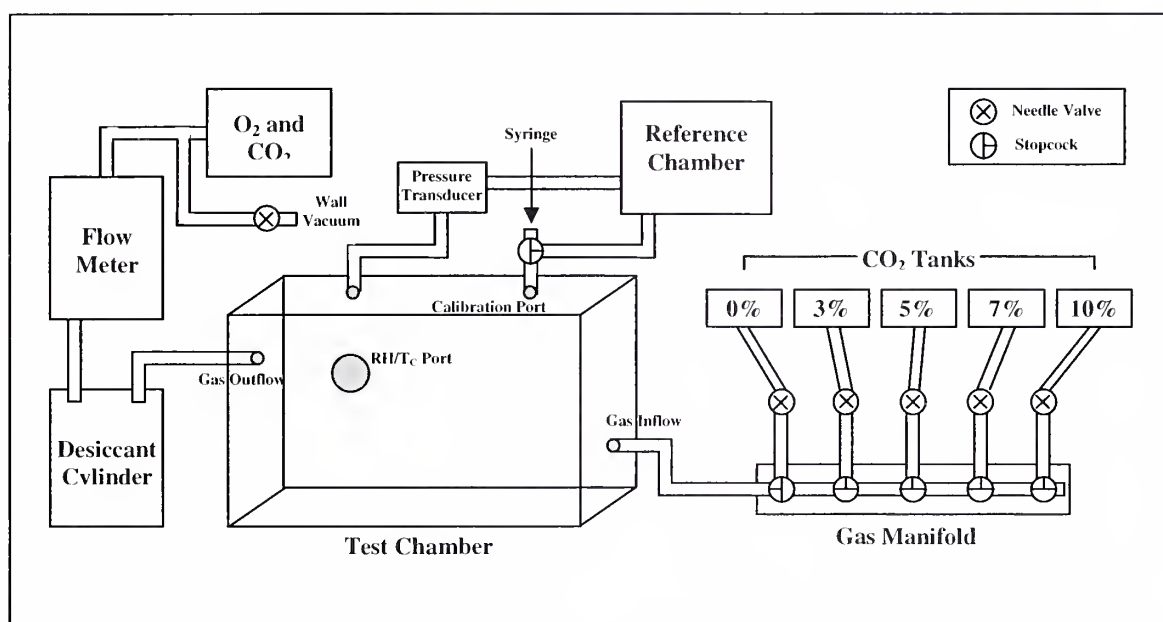


Figure 2 (a)

When placed in the plethysmography chamber each mouse was then exposed to a set protocol of gases [Figure 2 (b)]. Each exposure was set at ten minutes to allow the animal's breathing to stabilize while minimizing fatigue. Ten minutes at room air (21% O₂, balance Nitrogen) was followed by ten minute exposures to CO₂ mixes containing 3%, 5%, 7%, and 10% CO₂ respectively with 21% O₂ and balance Nitrogen. Every CO₂ exposure was alternated with ten minutes of room air exposure to allow for multiple sampling of the baseline ventilation and prevent respiratory fatigue secondary to prolonged hypercapnic exposure. Connected in series with the plethysmography

chamber was an O₂ and CO₂ analyzer which could verify the achievement of a stable CO₂ exposure level. The mouse was continually monitored throughout the experiment to maintain a state of quiet wakefulness, as sleep has an effect on the ventilatory response to hypercapnia (72, 73). Ten second time periods of quiet breathing were noted throughout the experiment to ensure usable data and marked for further analysis. At the end of each set of plethysmography experiment a 1cc syringe was used to repeatedly inject 0.1cc of air into the chamber with and without the mouse. This established a known volume to voltage relationship which could then be used to calculate absolute tidal volumes for each mouse.

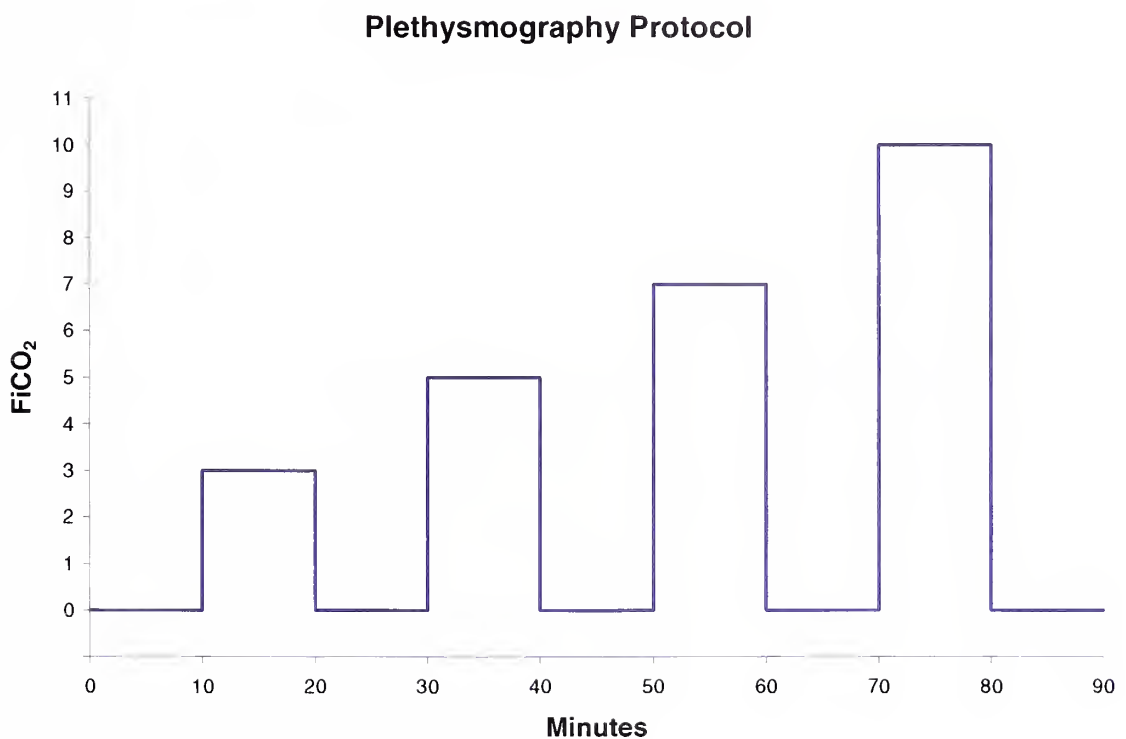


Figure 2 (b)

Hormone Exposure

In order to test the hypothesis that the deficiency seen in the ventilatory response of the male mice was a result of the relative lack of female sex hormones, the male mice were supplemented with estrogen and progesterone in the following manner. Each mouse served as its own control for these experiments, and was therefore studied in the plethysmography chamber as outlined above on Day 0, immediately prior to commencing hormone supplementation. The goal of hormone supplementation was to recapitulate as closely as possible the normal serum levels of both female sex hormones in the male mice, rather than administer a supra-physiologic dose. Normal female mice in their normal estrus cycle have a serum estrogen between 10 and 30 pg/ml (74), and serum progesterone between 10-60 ng/ml (75).

The adult male mice were treated with estrogen through their drinking water, as oral estrogen has good bioavailability. 17β -estradiol was dissolved at a 20 mM concentration in 95% ethyl alcohol (EtOH) and then diluted to a concentration of 2,500 nM in the standard chlorinated drinking water of the mice. Their daily water intake was then substituted with the estrogen drinking water. Preliminary supplementation in non-experimental mice had already been undertaken to show that Pet-1 mice drank the supplemented water and achieved their daily intake needs without dehydration. Estrogen drinking water at 2,500 nM dose has been shown to correspond to an estrogen serum level of 38 pg/ml – at the high range of estrogen throughout the normal mouse estrus cycle (76). Each experimental mouse had access to the estrogen drinking water for 48 hours, immediately following their control protocol in the plethysmography chamber.

Stable plasma levels of estrogen would be expected within six hours of oral administration, well within the exposure duration for this experiment (77).

As progesterone is extensively metabolized by the liver on first pass and therefore has poor oral bioavailability it is most commonly administered by intramuscular injection or subcutaneous capsules. Although progesterone has a half-life in circulation of only 5 minutes, suspension in sterile sesame oil allows for a depo-like effect with slowly declining serum levels after a single injection or implantation (78, 79). Experiments in humans have shown that injection at a dose of 1.5 mg/kg achieves a serum level between 5 and 50 ng/ml over 48 hours (78), slightly lower than the normal level in mice. The male mice therefore received a subcutaneous injection of 2 mg/kg of progesterone suspended in 0.1 mL of sterile sesame oil one hour before the plethysmography experiment.

It was noted during the course of these experiments that following the injection some sesame oil would be expressed back out of the injection site, usually following vigorous motor activity. While this would have reduced the total progesterone dose received by the mouse, the pharmacokinetics of progesterone is such that the dose injected has less of an impact on peak plasma levels than on the duration of the elevation. In humans, a dose one quarter of the dose utilized in these experiments achieves similar peak plasma levels of progesterone, however, the duration of the elevation is shortened from 24 hours to 12 hours (78). Qualitative assessment after depositing 0.1 mL of sesame oil directly onto the skin of a mouse suggested that much greater than 50% of the injected dose remained subcutaneous throughout the experiment. As the experiments

lasted for approximately 3 hours post-injection, even 50% of the injected dose would produce target plasma levels for many hours longer than required.

Data Analysis

The voltage vs. time data was recorded for each experiment in MatLab file format, according to the previously described protocol. Another custom-designed MatLab 7.0.2 was used to access this file and display it in plotted form, where the data could be explored for ten second segments of quiet breathing, without mouse movement, sniffing, or sighs. Breaths during the segment were identified by the peaks and troughs of the voltage, and the interspike interval and amplitude was averaged across all breaths in the segment. This procedure was repeated across all CO₂ exposure levels with the goal of finding at least six ten second segments of useable data during each gas exposure. The mean interbreath interval and amplitude along with standard deviations were then calculated for each gas exposure by averaging the values from the ten second segments. The final room air interbreath interval and amplitude was obtained by an average of all five ten minute exposures to minimize the effect of escalating CO₂ on baseline ventilation. The volume calibration performed with the mouse in the chamber was then used to assist calculations of the absolute tidal volumes from the average voltage deflection per breath in the following manner.

Since the pressure exerted by any gas varies with its temperature and partial pressure of H₂O, the air warmed and humidified by the lungs expands, and increases the pressure in the sealed chamber upon inhalation. The chamber temperature (T_c) and relative humidity (both measured parameters) are used to calculate the partial pressure of

water in the chamber (P_{cH_2O}). The animal's pulmonary temperature is assumed to be equal to the animal's body temperature (T_a), with the relative humidity of the inspired air equal to the saturation humidity at that temperature. Because the animal's temperature was not directly measured in these experiments, it was assumed to be 37° C for all animals and the water pressure of pulmonary air calculated (P_{aH_2O}). The known calibration volume (V_{cal}) injected into the chamber that causes a known pressure change (P_{cal}) was used to scale the following formula derived from the ideal gas law, where P_b is the ambient barometric pressure and where A equals the voltage deflection of the animal's breath (80):

$$V_T = A (V_{cal} / P_{cal}) [T_a(P_b - P_{cH_2O})] / [[T_a(P_b - P_{cH_2O})] - [T_c(P_b - P_{aH_2O})]]$$

As the frequency of respiration is easily generated from the interbreath interval (Frequency = 1 / IB Interval), absolute ventilation (V_E) can be calculated by taking the frequency multiplied by tidal volume.

$$V_E \text{ (mL / minute)} = \text{Frequency} * V_T$$

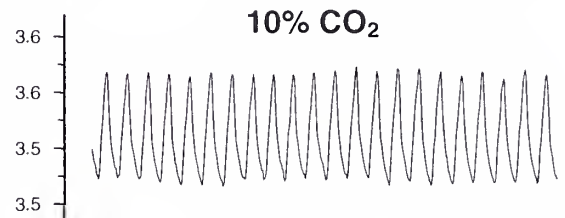
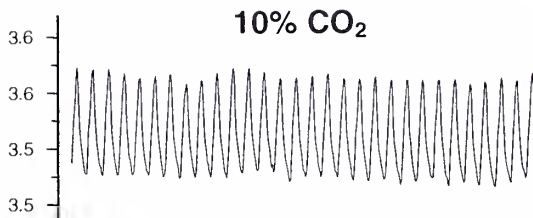
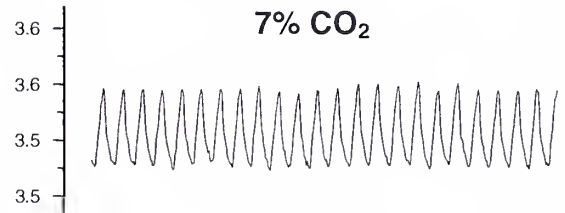
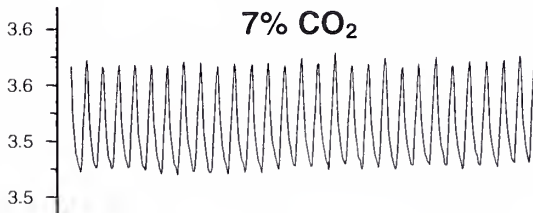
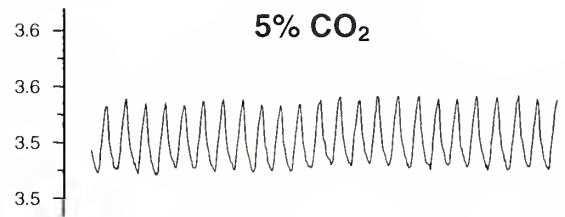
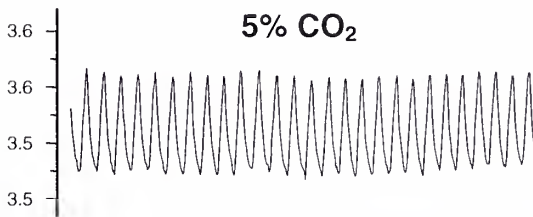
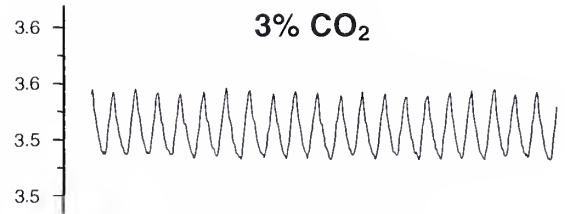
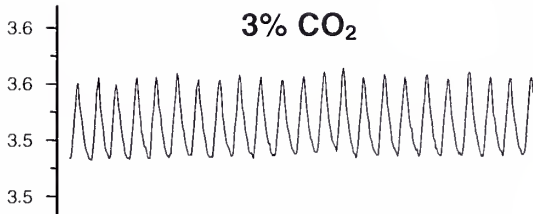
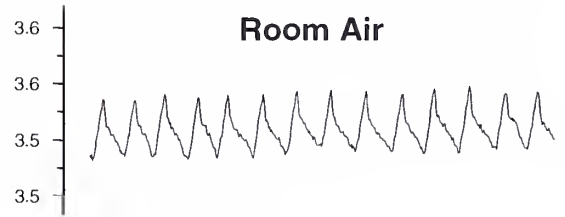
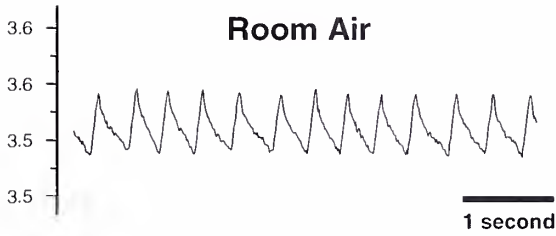
Since the absolute tidal volume (mL) depends on the size of the mouse and the relative size of its lungs, pooling data from all mice require that both V_T and V_E be scaled. A surrogate and more easily measured variable for total lung capacity is the weight of the mouse. V_T and V_E are therefore reported as adjusted for mass - V_T (mL / g) and V_E (mL / minute/ g).

For every mouse a respiratory frequency, V_T , and V_E were calculated for each ten minute gas exposure (Room Air, 3%, 5%, 7%, and 10% CO_2). As explained above, in order to minimize variability in the control measurement as a result of escalating CO_2 , the final control frequency, V_T , and V_E were obtained by an average of all five control exposures. These results were then pooled and sorted by genotype with statistical significance determined by the Paired Student's T-test.

Results

Consistent with the previous experiments on Pet-1 mice, a deficiency in their ventilatory response to normoxic hypercapnia was seen across multiple CO_2 levels. Seen side-by-side, the ventilatory tracing of a Pet-1 knockout mouse can be readily distinguished from a wild-type mouse across multiple CO_2 exposures [Figure 3].

Before hormone treatment each genotype's ventilatory response was characterized according to tidal volume, frequency, and ventilation (V_E) [Figure 4 (a), (b), (c)]. Little difference was seen in tidal volume between the wildtype versus knockout – at Room Air (1.54 ± 0.08 vs. 1.37 ± 0.11 ml/g), 3% CO_2 (2.00 ± 0.18 vs. 1.72 ± 0.13 ml/g), 5% CO_2 (2.18 ± 0.16 vs. 1.83 ± 0.17 ml/g), 7% CO_2 (2.31 ± 0.16 vs. 2.13 ± 0.10 ml/g), and 10% CO_2 (2.48 ± 0.17 vs. 2.49 ± 0.12 ml/g) these differences were not statistically significant ($p > 0.05$ for all data points).

Wild Type**Pet-1 Knockout****Figure 3**

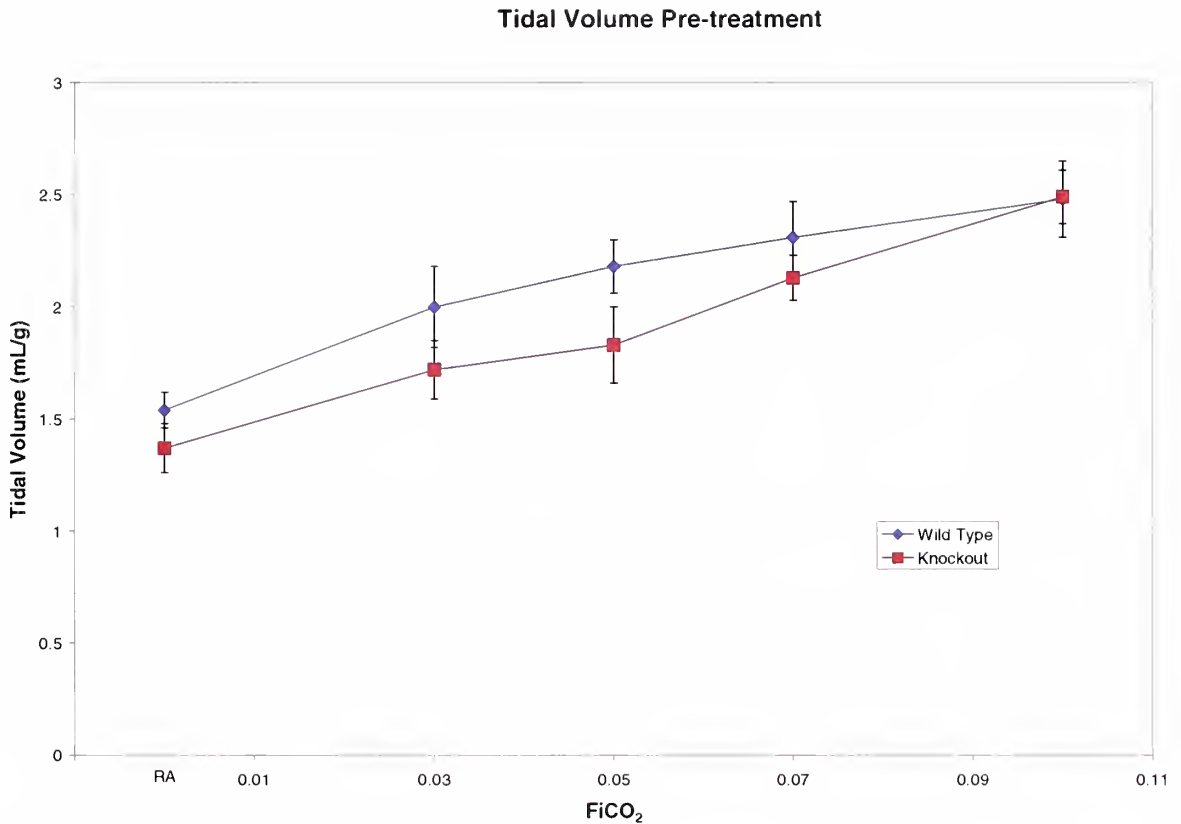


Figure 4 (a)

A different result was seen for frequency. Across most of the range of CO₂ exposure there was a statistically significant difference between the wildtype and knockout mice. At 3% CO₂ (282 ± 10 vs. 242 ± 8 breaths/minute; $p < 0.05$), 5% (301 ± 14 vs. 260 ± 8 breaths/minute; $p < 0.05$), and 7% (315 ± 14 vs. 270 ± 11 breaths/minute; $p = < 0.05$) the difference in frequency was statistically significant. As would be expected if the baseline ventilation was unaffected, the control respiratory frequency was not statistically significant between the two groups (179 ± 10 vs. 167 ± 10 breaths/minute; $p = \text{NS}$). Interestingly, the breathing frequency was similar between the two groups at 10% CO₂ as well (308 ± 12 vs. 290 ± 7 breaths/minute; NS).

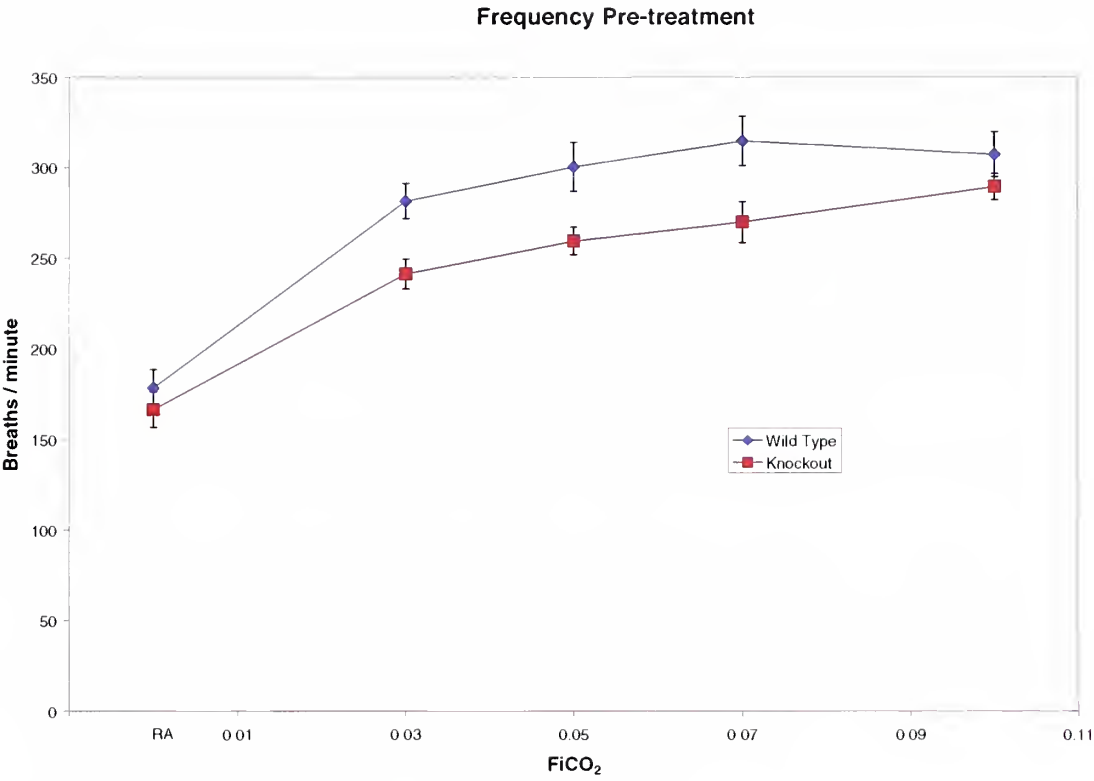


Figure 4 (b)

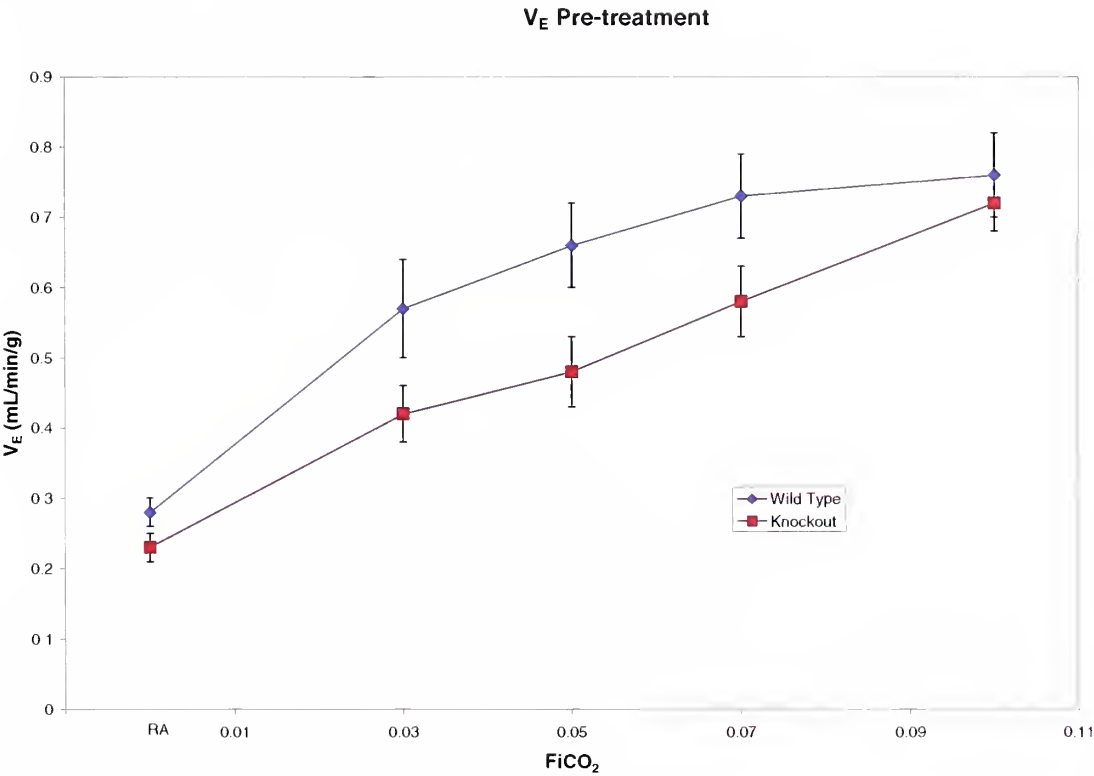


Figure 4 (c)

The difference in respiratory frequency therefore accounts for most of the difference in overall ventilation between the two groups. There was a trend towards lower overall ventilation under control conditions in the knockouts (0.28 ± 0.02 vs. 0.23 ± 0.02 ml/min/g; NS). The same trend was noted across all gas exposures up to 10% CO₂ although it achieved statistical significance only at 5% CO₂: 3% CO₂ (0.57 ± 0.07 vs. 0.42 ± 0.04 ml/min/g; NS), 5% CO₂ (0.66 ± 0.06 vs. 0.48 ± 0.04 ml/min/g; $p < 0.05$), and 7% CO₂ (0.73 ± 0.06 vs. 0.58 ± 0.05 ml/min/g; NS). At 10% CO₂, the knockout's ventilatory response has clearly recovered to equal that of the wildtype (0.76 ± 0.06 vs. 0.72 ± 0.04 ml/min/g; NS). This result could suggest that mechanisms other than the serotonin system could assist in the ventilatory response under extreme duress and will be more fully discussed later.

Graphing the results as percent change from the control exposure gives a different perspective on these results [Figure 4 (d)]. The hypercapnic ventilatory response (HCVR) is usually determined from the slope of the CO₂ response across multiple CO₂ levels ($\Delta V_E / \Delta F_I \text{CO}_2$). As seen from the % Response curve for the wildtype and knockout mice each genotype has a change in ventilation from their baseline, although as discussed previously, the absolute ventilation across multiple carbon dioxide levels trends lower at all hypercapnic exposures other than 10% CO₂.

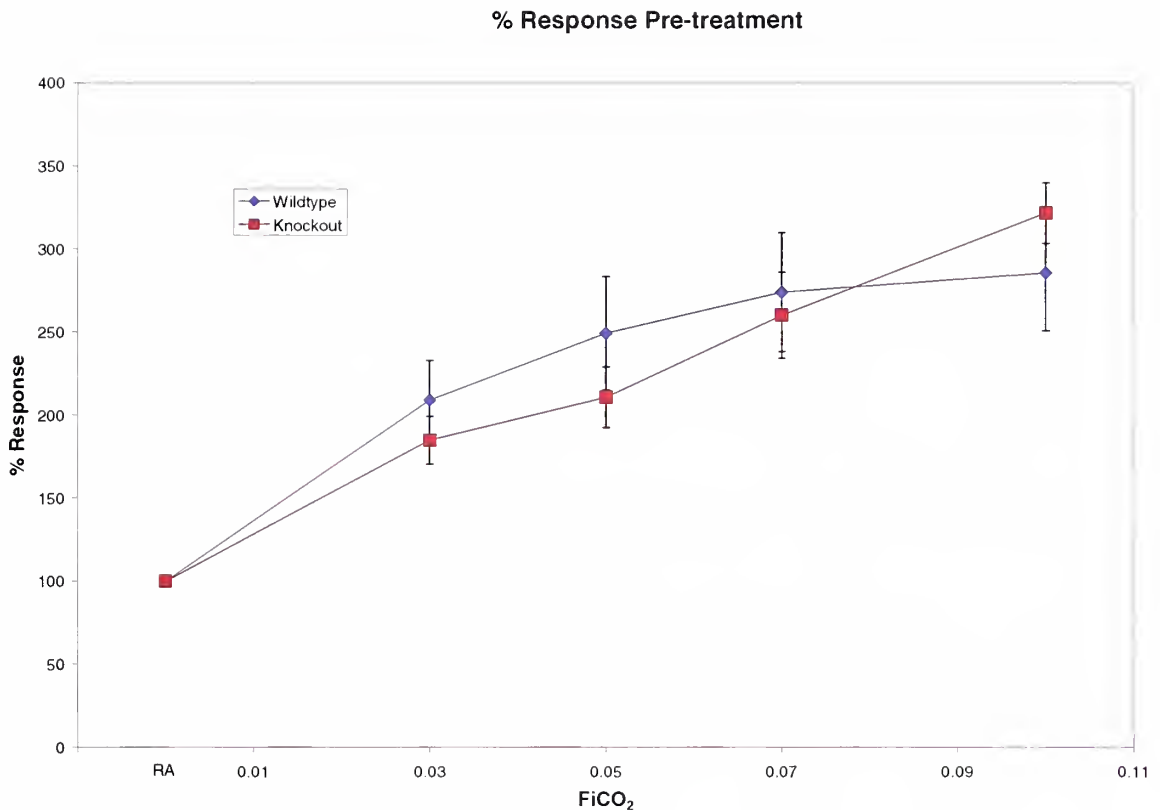


Figure 4 (d)

Hormone Therapy

After acute treatment with hormone therapy the same ventilatory parameters were measured again [Figure 5 (a), (b), (c)].

As seen before hormone therapy, there was no difference between the wild type and knockout groups in tidal volume, nor were the tidal volumes increased consistently as a result of the hormones. At all exposures there was not a statistical difference between the groups: control (1.48 ± 0.05 vs. 1.35 ± 0.07 ml/g; NS), 3% CO₂ (1.88 ± 0.07 vs. 1.62 ± 0.09 ml/g; NS), 5% CO₂ (1.95 ± 0.09 vs. 1.95 ± 0.1 ml/g), 7% CO₂ (2.07 ± 0.12 vs. 2.20 ± 0.12 ml/g), and 10% CO₂ (2.29 ± 0.1 vs. 2.50 ± 0.15 ml/g).

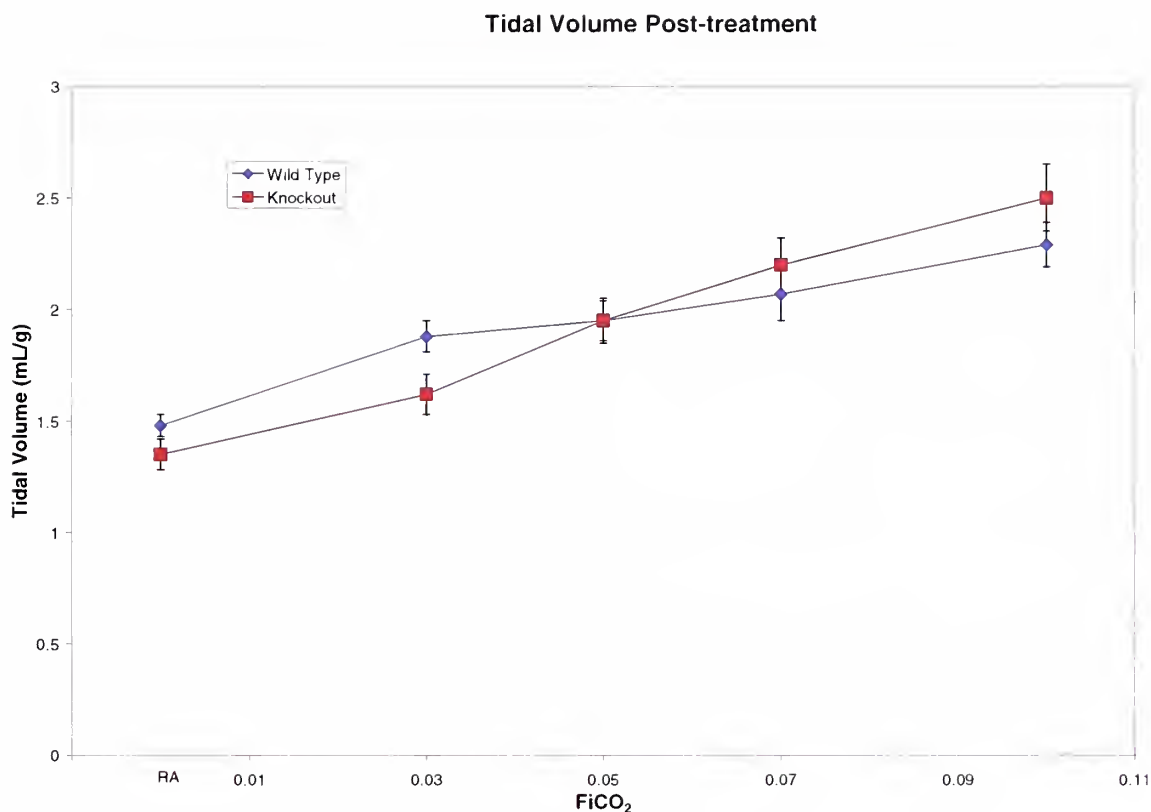


Figure 5 (a)

Similar to the results seen before hormone therapy, the ventilatory frequency was the respiratory parameter that showed the most difference between the two groups. At room air (195 ± 8 vs. 154 ± 11 breaths/minute; $p < 0.05$) and all carbon dioxide exposures up to 7% CO_2 there was a significant difference between the two genotypes: 3% CO_2 (294 ± 7 vs. 225 ± 10 breaths/minute; $p < 0.001$), 5% CO_2 (300 ± 10 vs. 248 ± 6 breaths/minute; $p < 0.005$). At 7% CO_2 (308 ± 11 vs. 282 ± 13 breaths/minute; NS) and 10% CO_2 (307 ± 9 vs. 278 ± 13 breaths/minute; NS) there was no statistical significance between the groups. Therefore, hormone therapy with estrogen and progesterone did not alter the basic characteristics of the differences in hypercapnic ventilatory response between the two genotypes.

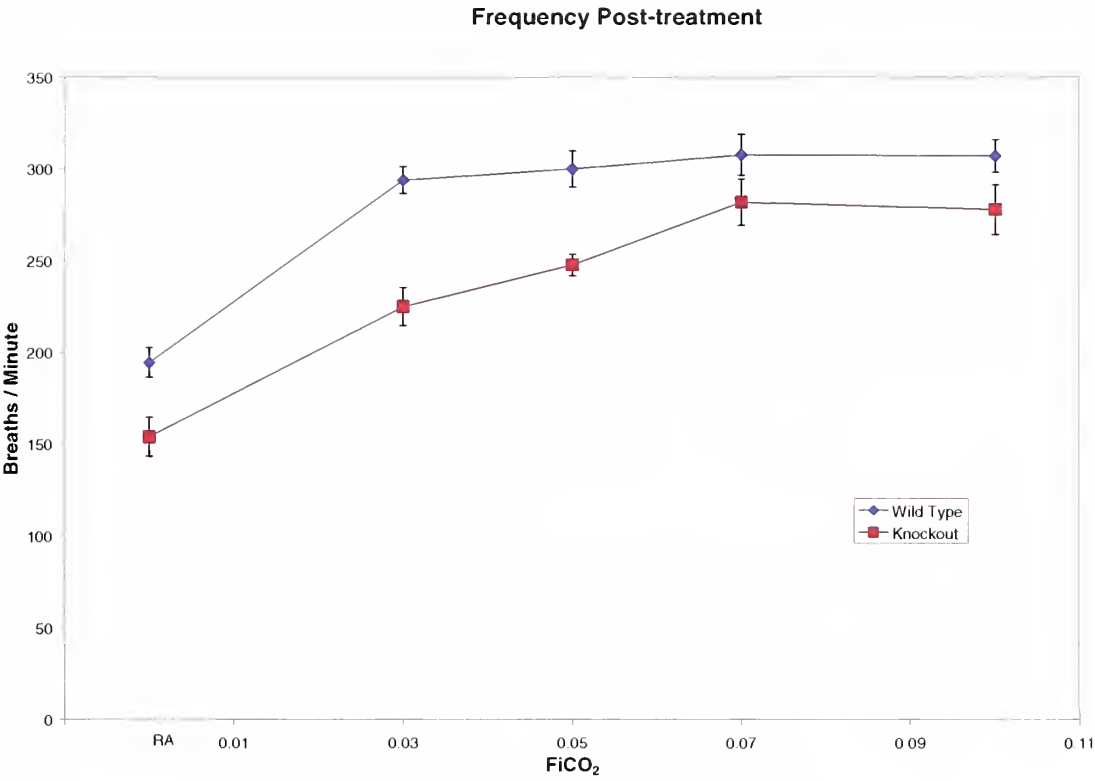


Figure 5 (b)

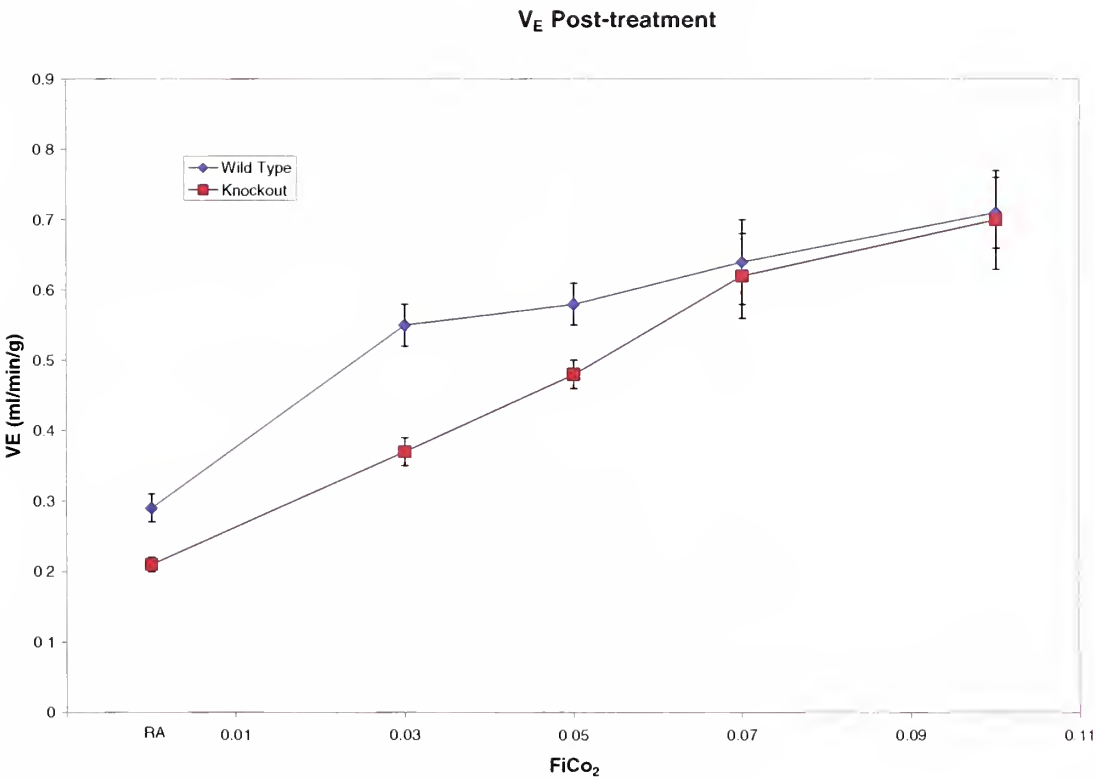


Figure 5 (c)

Total ventilation continued to be reduced in the knockout mice at control and lower CO₂ levels even after treatment with estrogen and progesterone, although the ventilation recovery seen in the knockout mice at the highest CO₂ exposure was now obvious at both 7% and 10% CO₂. The difference between the groups was statistically different at room air (0.29 ± 0.02 vs. 0.21 ± 0.01 ml/min/g; $p < 0.005$), 3% CO₂ (0.55 ± 0.03 vs. 0.37 ± 0.02 ml/min/g; $p < 0.0005$), and 5% CO₂ (0.58 ± 0.03 vs. 0.48 ± 0.02 ml/min/g; $p < 0.05$). After treatment total ventilation at both 7% CO₂ (0.64 ± 0.06 vs. 0.62 ± 0.06 ; NS) and 10% CO₂ (0.71 ± 0.05 vs. 0.70 ± 0.07 ml/min/g; NS) was not statistically different. This result could suggest that the mechanism that responds to higher levels of hypercapnic stress is hormone sensitive.

This finding is emphasized by a plot of the ventilatory response compared as a percent to the baseline room air breathing [Figure 5 (d)]. Before treatment the knockout group had a similar slope to the wildtype, but under the influence of female hormones it shows a more robust response, especially at the higher CO₂ levels. However, this trend was not statistically significant: 7% CO₂ (227 ± 27 vs. 305 ± 33 % response; NS) and 10% CO₂ (250 ± 25 vs. 344 ± 41 % response; NS).

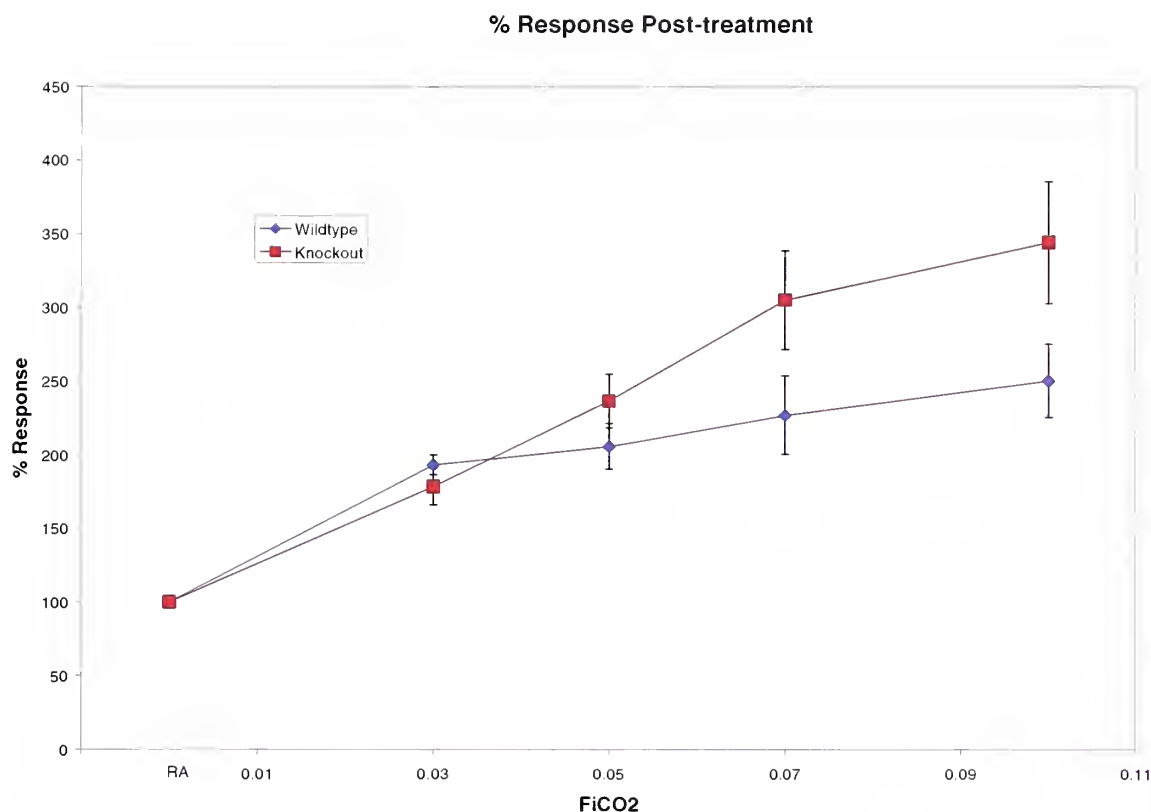


Figure 5 (d)

When comparing the absolute ventilation with and without hormones there are little differences between the wildtype or knockout groups to suggest that absolute ventilation changed as a result of hormone treatment [Figure 5 (e)]. At room air and across all CO₂ exposures there was no significant change in V_E in the wildtype group: room air (0.28 ± 0.02 vs. 0.29 ± 0.02 ml/min/g), 3% CO₂ (0.57 ± 0.07 vs. 0.55 ± 0.03 ml/min/g), 5% CO₂ (0.66 ± 0.06 vs. 0.58 ± 0.03 ml/min/g), 7% CO₂ (0.73 ± 0.06 vs. 0.64 ± 0.06 ml/min/g), and 10% CO₂ (0.76 ± 0.06 vs. 0.71 ± 0.05 ml/min/g). Therefore it does not appear that this level of acute supplementation with female hormones stimulated breathing in the wildtype mice.

Similarly there was no difference in absolute ventilation in the knockout group as a result of hormone treatment when directly compared to the pretreatment ventilation: room air (0.23 ± 0.02 vs. 0.21 ± 0.01 ml/min/g), 3% CO₂ (0.42 ± 0.04 vs. 0.37 ± 0.02 ml/min/g), 5% CO₂ (0.48 ± 0.05 vs. 0.48 ± 0.02 ml/min/g), 7% CO₂ (0.58 ± 0.05 vs. 0.62 ± 0.06 ml/min/g), and 10% CO₂ (0.72 ± 0.04 vs. 0.70 ± 0.07 ml/min/g).

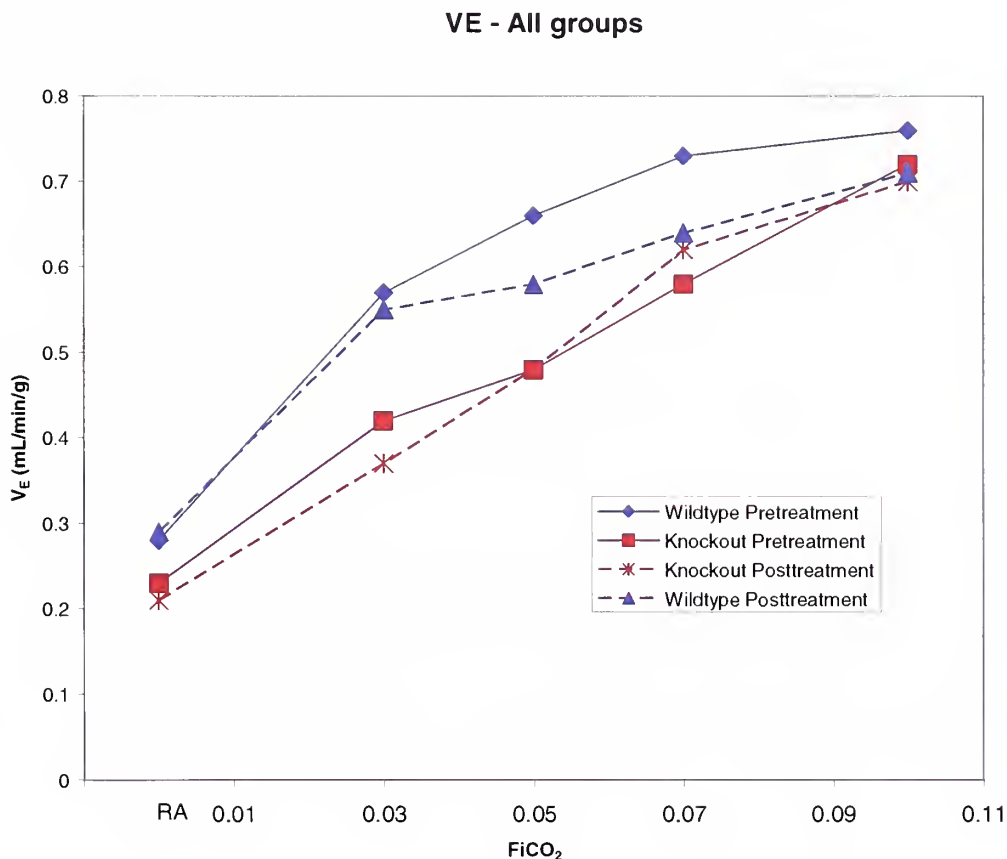


Figure 5 (e)

The higher percentile response seen after hormone treatment in the knockout group is due to a combination of factors – a slight decrease in the room air ventilation for the knockout group versus a slight increase the wildtype, along with a small *decrease* in the overall response of the wildtype that magnifies the effect of an identical response in

the knockout group. Therefore it can be concluded that supplementation with female sex hormones did not significantly impact the absolute ventilation of either the wildtype or knockout Pet-1 mice, but there is evidence that it changed the relative response between the groups at higher CO₂ exposures.

Discussion

The goals of this study were to 1) confirm and further define the deficiencies of the Pet-1 male's response to normoxic hypercapnia and to 2) determine if this deficiency could be accounted for and corrected by physiologic supplementation with female sex hormones.

In a confirmation of previously obtained results, the Pet-1 knockout mice showed a statistically significant deficiency in their total ventilation, mainly at lower hypercapnic exposures. The trend seen pre-treatment did not achieve statistical significance except at 5% CO₂ most likely because of the small sample size. However, as their room air ventilation was also depressed relative to the wildtype mice, their ventilatory response seen as a percentage of baseline was not different than the respiratory response of the wildtype. This raises the important question of the most accurate determinant of the hypercapnic ventilatory response. The HCVR is most commonly expressed as the slope of \dot{V}_E , which takes into account both the baseline ventilation and the hypercapnic ventilation; by this measure the Pet-1 knockout mice do not show a difference in their HCVR.

Despite this result, there is a clear phenotype for the male Pet-1 knockout mice compared to their wildtype kin. Their total ventilation is reduced both at room air and for all hypercapnic exposures except 10% CO₂. As is readily evident from the V_E graph, the decrease from the wildtype ventilation is not equivalent across all exposures, as might be expected if the loss of serotonergic neurons interfered with the fundamental mechanisms of breathing such as respiratory pattern generation, phrenic discharge, or diaphragmatic function. At room air, the knockout's V_E is 82% that of wildtype, 74%, 73%, and 79% at 3%, 5% and 7% CO₂ respectively and finally 95% at 10% CO₂. These variable results argue strongly that the disruption of the serotonin system in the Pet-1 mice does have an effect on the hypercapnic ventilatory response, but also that this effect is not uniform across the possible range of hypercapnic stressors. It could be concluded therefore, that the serotonin system plays an important role in maintaining baseline ventilation and the response to CO₂ at lower exposure levels, and that a secondary hypercapnic response independent of the serotonin system can assist in driving ventilation at higher carbon dioxide stresses.

There is a precedent for this result in other knockout genotypes also deficient in the serotonin system. Mice deficient in Lmx1b (a transcription factor necessary for neuronal development (81) driven by the Pet-1 promoter are more severely deficient in serotonin neurons than the Pet-1 knockout mice. These mice lack up to 99% of their serotonergic neurons in all serotonin nuclei. When tested in whole body plethysmography in a fashion similar to the Pet-1 mice, although using hyperoxic hypercapnia to disable peripheral chemoreceptors rather than normoxic hypercapnia, these mice show a more profound deficiency in their HCVR across a range of CO₂

similar to the results reported here [Figure 6]. The slope of V_E is linear (representing the HCVR) until 10% CO_2 , where a more robust ventilatory response is seen. This biphasic response suggests that the serotonin system is functioning as a central chemoreceptor responsible for driving a ventilatory response to CO_2 , but also that secondary neural systems can “rescue” the response under severe enough acidosis.

Lmx1b Knockout

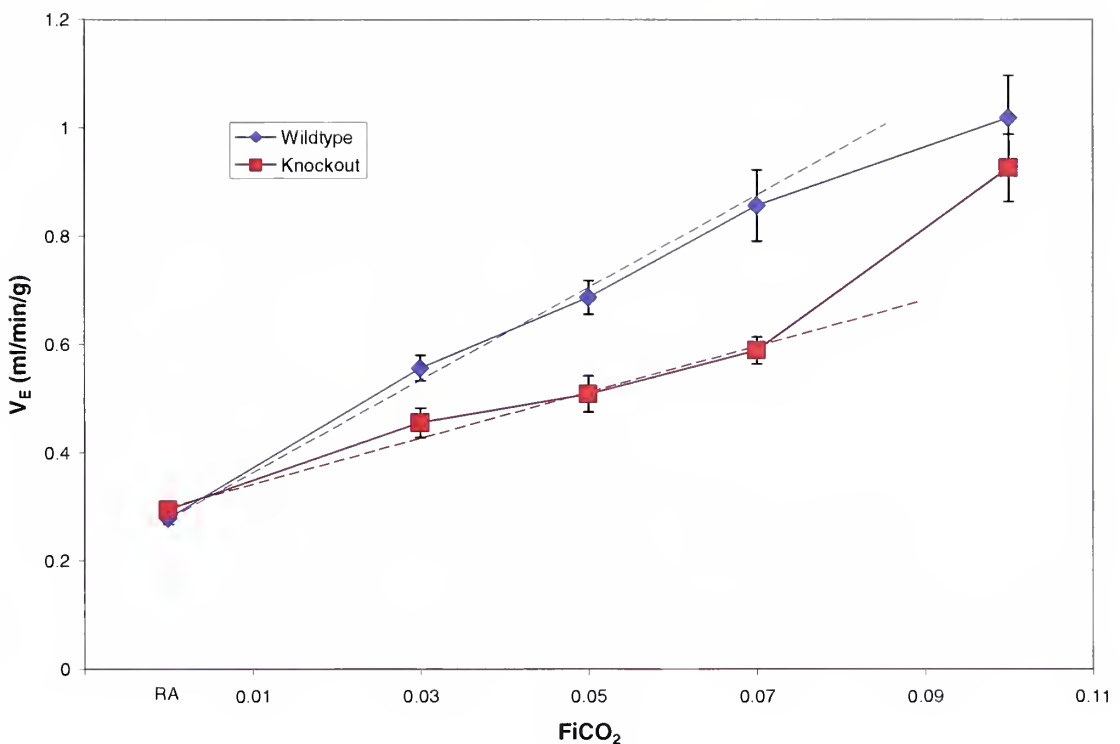


Figure 6

Seen in this context, the results from the current study support the theory of the serotonin system as a central chemoreceptor, albeit one whose effect is not constant across the entire range of hypercapnic challenges. However, the 70% loss of serotonin neurons appears to have only resulted in a ~25% loss of ventilatory response. What

could account for this difference if the hypothesis that serotonin contributes to the HCVR is accepted?

First, the experiment performed here took place under normoxic conditions. As has been well-described, peripheral chemoreceptors located in the carotid body provide the majority of the ventilatory response to hypoxia, but they also modulate the response to hypercapnia through changes in the parasympathetic or sympathetic systems and also respond directly to high CO₂ challenges (82, 83). Performing these experiments under hyperoxic conditions would decrease the influence of the peripheral chemoreceptors and might therefore unmask more of the Pet-1 knockout phenotype.

Second, there is no reason to presuppose that the phenotypic response should be linear ie) a 70% loss of serotonergic neurons would lead to a 70% reduction in the hypercapnic response. In fact, there are good reasons to suppose otherwise. 1) Although a 70% reduction in the number of serotonin neurons is quantitatively impressive, this does not address the capacity of the remaining 30%. These remaining neurons could be simply sufficient on their own to drive a ventilatory response or they could upregulate key transporters or enzymes as a compensatory response. Work with other neurotransmitter systems has shown that a surprisingly small number of remaining neurons can support essential functions. For example, in patients with unilateral Parkinson's Disease, imaging of the unaffected contralateral side shows a loss of up to 40% of the dopamine neurons (84). 2) Chemosensitivity is an extremely important homeostatic mechanism, and in the absence of a normal serotonin system it is possible that other neurotransmitter systems that normally have a minor role in chemosensitivity could be recruited for this function. Other candidate systems could include neurons of

the locus coeruleus (85-87), retrotrapeziod nucleus (RTN) (88, 89) or the nucleus tractus solitarius (NTS) (90, 91). 3) There are also inherent difficulties in interpreting the results of knockout mice. Soon after the development of homologous recombination to eliminate target genes, many knockout mice were generated that had surprisingly normal phenotypes given the thought-to-be essential nature of the target gene (92). In many cases this phenotypic normality has been attributed to gene redundancy, recruitment of alternative systems for compensation, or the superfluous nature of the target gene. This has led to the current popularity of “conditional knockouts”, where the temporal control over a gene allows for normal protein function during development, thus preventing accessory or redundant systems from compensating when the protein is removed in adult life (93). While the *Pet-1* mice do exhibit a clear neuropathologic phenotype, it is not clear what the effect of a reduction of serotonin neurons during fetal and early development has on normal brain function.

It should be noted that every one of these caveats argues for a normalization of phenotype, and therefore the ~25% reduction seen in ventilation and its differential relationship to hypercapnia is an extremely strong argument for the role of serotonin neurons in normal central chemosensitivity.

Sex Hormones and Chemosensitivity

The second goal of these experiments was to address the ability of female sex hormones to account for the differences seen between male and female *Pet-1* knockout mice. A combination of estrogen and progesterone was used based upon work in rats

discussed earlier that showed the need for both hormones to increase ventilation in males and females.

A comparison of pre and post-hormone treatment total ventilation (V_E) provides no evidence that the baseline ventilation of either the wildtype or the Pet-1 knockout group was affected by hormone therapy. This result is surprising given the extensive evidence that progesterone is a respiratory stimulant. Unfortunately, as there is no consensus about dosing or administration of these hormones, many of the experiments that show the stimulatory effects of progesterone use supra-physiologic doses.

In the current study progesterone was dosed at 2 mg/kg in an attempt to recapitulate normal female hormonal levels. In comparison, stimulation of ventilation in female rats was achieved with MPA dosed at 8 mg/kg x 4 days (64). In order to stimulate ventilation in male rats, they were fed TZP4238 (a progesterone analog with a potency 300-400 times that of progesterone) at 4 mg/kg, even though some respiratory effects were seen at 0.4 mg/kg (65). For the purposes of the current study, proving the ability of supra-physiologic progesterone to stimulate ventilation in Pet-1 mice would have had no relevance on the experimental question. Clearly, if the female Pet-1 knockout mice are protected by circulating sex hormones these hormones can provide adequate protection within physiologic ranges.

While the baseline ventilation was not affected, the sex hormones did appear to influence the ventilatory response at higher CO₂ exposures. The basic pattern seen pre-treatment, where ventilation at Room Air and lower CO₂ exposures was diminished, was retained only until 5% CO₂ – after hormone treatment the knockout's V_E was 72%, 67%, and 83% that of wildtype at Room Air, 3%, and 5% respectively. The ventilatory

recovery was 97% and 99% at 7% and 10% CO₂, although the caveats discussed earlier still apply – this difference was due to a small size in the absolute ventilation of the knockout along with a small decrease in the V_E of the wildtype.

If this recovery is real and due to the hormone exposure, there are two possible interpretations of this result: 1) The deficiency in the HCVR seen in the knockout mice as a result of the loss of serotonin neurons can be influenced by female sex hormones, or 2) secondary chemoreceptive mechanisms are influenced by female sex hormones and therefore “kick in” at earlier hypercapnic stresses. These are by no means mutually exclusive, and teleologically the rescue at 7% CO₂ could be explained either way. If the remaining serotonergic neurons were enhanced by female sex hormones, this effect would surely be seen when the neurons were receiving maximum extrinsic stimulation ie) at the highest CO₂ exposure. Similarly, redundant chemoreceptive neurons could be recruited at lower and lower CO₂ thresholds if they were responsive to sex hormones. Neither of these options can be ruled in or out at this point, so it can therefore be cautiously concluded that there is evidence that supplementation with physiologic levels of female hormones can restore some of the ventilatory deficiencies seen as a result of the loss of serotonin neurons.

Now that there is some experimental evidence that estrogen and progesterone can manipulate the phenotype caused by reduced serotonergic neurons it is reasonable to ask if there are other lines of evidence to support this interaction. Clear connections between the serotonergic system and sex hormones could tip the explanatory balance towards serotonergic neurons and away from the unknown redundant chemoreceptors.

Due in large part to the role of the serotonin system in psychiatric disease – mainly depression and anxiety, both of which affect females more than males (94) – the intersection between the serotonin system and sex hormones is an active area of research (95).

Estrogen and progesterone act through nuclear receptors – estrogen receptors in two major isoforms ER α and ER β (96) and progesterone isoforms PR-A and PR-B (97). In most species studied thus far including monkey, guinea pig, and mice, ER β is the form that is expressed most highly in the dorsal raphe. In mice, both isoforms colocalize with serotonin neurons (98), although ER β has a higher rate of colocalization in the dorsal raphe (90%) than ER α (23%) in male mice (99). Progesterone receptors in the serotonergic neurons are upregulated by ER β activation in both mice and monkeys and downregulated by progesterone (100, 101).

What is known about the effects on the serotonin system of activating these receptors? One key measure of serotonin activity is the level of TPH, which is the rate-limiting enzyme in the production of serotonin. When activated by estrogen, serotonin neurons upregulate TPH mRNA in both guinea pigs (102) and macaques (103), and the same effect has recently been shown in mice to be mediated through ER β (104). Whether this increase in mRNA results in increased TPH protein is not yet known in mice, but has been shown to be the case in guinea pigs and macaques (102, 103).

Another regulatory target of estrogen and progesterone could be SERT, the serotonin reuptake transporter. Treatment with estrogen increased SERT mRNA in the dorsal raphe of rats (105) although the opposite effect (decreased SERT mRNA) was seen in macaques (106). The physiologic effect of transport regulation is unclear

however, since serotonin binding and uptake can be increased even the face of decreased SERT mRNA (106). In mice, a low estrogen state led to a reduced density of SERT in the hippocampus but a paradoxical increase in SERT activity (107). Other possible targets for protein regulation like the 5HT1A autoreceptor (108) or MAO-A (109) show responses to various combinations of estrogen and progesterone.

It can therefore be concluded that serotonin neurons have the basic properties necessary to respond to female sex hormones – they express the appropriate receptors and have the ability to regulate both mRNA and protein in response to these signals. It is equally clear however, that invoking sex hormones to account for the difference in the Pet-1 knockout mice requires that sex hormones be able to modulate serotonergic neural responses.

Accordingly, a wide range of functions in which serotonin is implicated have been shown to be modulated by female sex hormones (110). The most basic of these is a difference in the tonic activity of dorsal raphe serotonin neurons between the sexes. In male rats, serotonergic neurons were found to have an average firing rate 41% higher than normal and ovariectomized females that was *not* correlated with circulating levels of progesterone and estrogen. During pregnancy, the basal firing rate of the females increased up to 136%, and this rise and subsequent fall late in pregnancy was closely reflected in the circulating progesterone level (111). Further work by the same group established that these differences were due to a partial desensitization of the 5-HT1A receptors during pregnancy (112). Stimulation of the basal firing rate was achieved with intra-cerebral ventricular DHEA, progesterone, and its metabolites in the females (113), but these effects were not seen in males. Instead, the baseline rate in both males and

females was modulated by testosterone as well as with estrogen (which lies downstream from testosterone in metabolism) (114).

It would be imprudent to attempt to summarize all other actions of female sex hormones on the serotonin system here, suffice it to say that their ability to act on it is well-established (110).

The specific function in question here is the role of sex hormones on the serotonin system as it pertains to breathing. There is not much experimental evidence in this area, apart from the current study, the only work to address this question measured serotonin levels in respiratory nuclei in male and female rats throughout their estrus cycle (38). Females were found to have significantly higher levels of serotonin in the hypoglossal nucleus than the males, but equal levels in the phrenic nucleus. The serotonin levels in the different nuclei changed throughout the estrus cycle, with interesting results. In the hypoglossal nucleus, the changes in serotonin levels closely mirror plasma estrogen, while in the phrenic nucleus serotonin levels mirrored serum progesterone. These results are consistent with the current study, as downstream targets would be expected to show increased level of serotonin as the HCVR is influenced by hormonal status.

Given the enormous amount of evidence that links the serotonergic system with female sex hormones any evidence that the knockout phenotype of the Pet-1 male mice is altered by estrogen and progesterone must be taken seriously. The question remains however, if it accepted that estrogen and progesterone restored some of the remaining serotonergic function, why did they not restore all of the difference between the male and female Pet-1 knockout mice?

It is certainly possible that a higher dose of estrogen and progesterone could have shown a larger impact on the ventilatory response, but this would not have addressed the phenotypic difference between the sexes. Ovariectomy in the female mice would also have addressed this question, but is far more invasive than hormone supplementation in the males. Given the results of this study it is a logical follow-up experiment.

The most obvious explanation for the results is that acute supplementation with estrogen and progesterone in male mice cannot replicate the influence of these hormones throughout the neural and physiologic development of the female mice. There is support for this finding in studies of the sexual dimorphism of the serotonin system, where early in rat development (day E14) estrogen has effects on neurite growth (115). Within the second postnatal week, there are already sex differences in the serotonergic system in rodent brains (116). Klink et al hypothesize that the difference in the dorsal raphe basal rate response to estrogen and progesterone is a gender-dependent trait, as the ovariectomized female mice do not take on the male phenotype (111). If the difference seen in the female and male Pet-1 knockout mice is due to this sort of gender-dependent developmental mechanism then no amount of hormone supplementation would be able to fully restore the male phenotype.

In conclusion, the present study reports that male Pet-1 knockout mice have a variable ventilatory deficiency in response to a normoxic hypercapnic challenge, with a decreased HCVR at lower CO₂ exposures. This deficiency is partially reversed by supplementation with physiologic levels of female sex hormones, suggesting that the function of the remaining serotonergic neurons can be boosted by estrogen and

progesterone and providing a partial explanation for the sparing of the female Pet-1 knockout mice hypercapnic ventilatory response.

References

1. Richerson GB. Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. *Nat Rev Neurosci* 2004;5(6):449-61.
2. Mitchel RA, Loeschcke HH, Massion WH and Severinghaus JW. Respiratory responses mediated through superficial chemosensitive areas on the medulla. *J Appl Physiol* 1963;18:523-33.
3. Dahlstroem A, Fuxe K. Evidence for the existence of monoamine-containing neurons in the central nervous system: I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta Physiol Scand* 1964;62:SUPPL 232:1-55.
4. Lovick TA. The medullary raphe nuclei: a system for integration and gain control in autonomic and somatomotor responsiveness? *Exp Physiol* 1997;82(1):31-41.
5. Mason P. Contributions of the medullary raphe and ventromedial reticular region to pain modulation and other homeostatic functions. *Annu Rev Neurosci* 2001;24:737-77.
6. Jacobs BL, Fornal CA. Serotonin and motor activity. *Curr Opin Neurobiol* 1997;7(6):820-5.
7. Fuxe K. Evidence for the existence of monoamine neurons in the central nervous system: IV. Distribution of monoamine nerve terminals in the central nervous system. *Acta Physiol Scand* 1965;64:SUPPL 247:37+.
8. Voss MD, De Castro D, Lipski J, Pilowsky PM and Jiang C. Serotonin immunoreactive boutons form close appositions with respiratory neurons of the dorsal respiratory group in the cat. *J Comp Neurol* 1990;295(2):208-18.
9. Pilowsky PM, de Castro D, Llewellyn-Smith I, Lipski J and Voss MD. Serotonin immunoreactive boutons make synapses with feline phrenic motoneurons. *J Neurosci* 1990;10(4):1091-8.
10. Holtman JR, Jr. Immunohistochemical localization of serotonin- and substance P-containing fibers around respiratory muscle motoneurons in the nucleus ambiguus of the cat. *Neuroscience* 1988;26(1):169-78.
11. Holtman JR, Jr, Norman WP and Skirboll L, et al. Evidence for 5-hydroxytryptamine, substance P, and thyrotropin-releasing hormone in neurons innervating the phrenic motor nucleus. *J Neurosci* 1984;4(4):1064-71.
12. Manzke T, Guenther U and Ponimaskin EG, et al. 5-HT₄(a) receptors avert opioid-induced breathing depression without loss of analgesia. *Science* 2003;301(5630):226-9.

13. Garner SJ, Eldridge FL, Wagner PG and Dowell RT. Buspirone, an anxiolytic drug that stimulates respiration. *Am Rev Respir Dis* 1989;139(4):946-50.
14. Mendelson WB, Martin JV and Rapoport DM. Effects of buspirone on sleep and respiration. *Am Rev Respir Dis* 1990;141(6):1527-30.
15. Sahibzada N, Ferreira M, Wasserman AM, Taveira-DaSilva AM and Gillis RA. Reversal of morphine-induced apnea in the anesthetized rat by drugs that activate 5-hydroxytryptamine(1A) receptors. *J Pharmacol Exp Ther* 2000;292(2):704-13.
16. Lalley PM. Serotonergic and non-serotonergic responses of phrenic motoneurons to raphe stimulation in the cat. *J Physiol* 1986;380:373-85.
17. Haxhiu MA, Erokwu B, Bhardwaj V and Dreshaj IA. The role of the medullary raphe nuclei in regulation of cholinergic outflow to the airways. *J Auton Nerv Syst* 1998;69(1):64-71.
18. Mueller RA, Towle AC and Breese GR. Supersensitivity to the respiratory stimulatory effect of TRH in 5,7-dihydroxytryptamine-treated rats. *Brain Res* 1984;298(2):370-3.
19. Richerson GB. Response to CO₂ of neurons in the rostral ventral medulla in vitro. *J Neurophysiol* 1995;73(3):933-44.
20. Wang W, Pizzonia JH and Richerson GB. Chemosensitivity of rat medullary raphe neurons in primary tissue culture. *J Physiol* 1998;511 (Pt 2)(Pt 2):433-50.
21. Wang W, Tiwari JK, Bradley SR, Zaykin RV and Richerson GB. Acidosis-stimulated neurons of the medullary raphe are serotonergic. *J Neurophysiol* 2001;85(5):2224-35.
22. Wang W, Bradley SR and Richerson GB. Quantification of the response of rat medullary raphe neurons to independent changes in pH(o) and P(CO₂). *J Physiol* 2002;540(Pt 3):951-70.
23. Tiwari JK, Zaykin AV, Ua Cruadhlaioich MI, Wang W and Richerson GB. A novel pH sensitive cation current is present in putative central chemoreceptors of the medullary raphe. *Soc Neurosci Abstr* 2000;26(154.1).
24. Bradley SR, Pieribone VA, Wang W, Severson CA, Jacobs RA and Richerson GB. Chemosensitive serotonergic neurons are closely associated with large medullary arteries. *Nat Neurosci* 2002;5(5):401-2.
25. Haxhiu MA, Tolentino-Silva F, Pete G, Kc P and Mack SO. Monoaminergic neurons, chemosensation and arousal. *Respir Physiol* 2001;129(1-2):191-209.
26. Johnson PL, Hollis JH, Moratalla R, Lightman SL and Lowry CA. A panicogenic stimulus (acute hypercapnia) increases c-fos immunoreactivity in subpopulations of midbrain serotonergic neurons. *Soc Neurosci Abstr* 2003;29(712.5).

27. Veasey SC, Fornal CA, Metzler CW and Jacobs BL. Response of serotonergic caudal raphe neurons in relation to specific motor activities in freely moving cats. *J Neurosci* 1995;15(7 Pt 2):5346-59.
28. Bernard DG, Li A and Nattie EE. Evidence for central chemoreception in the midline raphe. *J Appl Physiol* 1996;80(1):108-15.
29. Nattie EE, Li A. CO₂ dialysis in the medullary raphe of the rat increases ventilation in sleep. *J Appl Physiol* 2001;90(4):1247-57.
30. Hodges MR, Opansky C and Qian B, et al. Transient attenuation of CO₂ sensitivity after neurotoxic lesions in the medullary raphe area of awake goats. *J Appl Physiol* 2004;97(6):2236-47.
31. Hodges MR, Martino P, Davis S, Opansky C, Pan LG and Forster HV. Effects on breathing of focal acidosis at multiple medullary raphe sites in awake goats. *J Appl Physiol* 2004;97(6):2303-9.
32. Messier ML, Li A and Nattie EE. Inhibition of medullary raphe serotonergic neurons has age-dependent effects on the CO₂ response in newborn piglets. *J Appl Physiol* 2004;96(5):1909-19.
33. Nattie EE, Li A, Richerson GB and Lappi DA. Medullary serotonergic neurons and adjacent neurons that express neurokinin-1 receptors are both involved in chemoreception in vivo. *J Physiol* (in press);.
34. Scott MM, Wylie CJ and Lerch JK, et al. A genetic approach to access serotonin neurons for in vivo and in vitro studies. *Proc Natl Acad Sci U S A* 2005;102(45):16472-7.
35. Hendricks TJ, Fyodorov DV and Wegman LJ, et al. Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. *Neuron* 2003;37(2):233-47.
36. Hodges MR, Best SR, Deneris ES and Richerson GB. Adult Pet-1 Knockout Mice Exhibit an Attenuated Hypercapnic Ventilatory Response. *Soc Neurosci Abstr* 2005;.
37. Saaresranta T, Polo O. Hormones and breathing. *Chest* 2002;122(6):2165-82.
38. Behan M, Zabka AG, Thomas CF and Mitchell GS. Sex steroid hormones and the neural control of breathing. *Respir Physiol Neurobiol* 2003;136(2-3):249-63.
39. Hasselbach KA. Ein Betrag zur Respirationphysiologie der Gradiditat. *Skand Arch Physiol* 1912;27:1-12.
40. Hasselbach KA, Gammeltoft SA. Die Nertralitätsregulation des graviden Organismus. *Biochem Z* 1915;68:206-64.

41. Plass ED, Oberst FW. Respiration and pulmonary ventilation in normal nonpregnant, pregnant, and puerperal women with an interpretation of acid-base balance during normal pregnancy. *Am J Obstet Gynecol* 1938;35:441-52.
42. Griffith FR, Pucker GW, Brownell KA, Klein JD and Carmer ME. Studies in human physiology, alveolar air and blood gas capacity. *Am J Physiol* 1929;89:449-70.
43. Goodland RL, Reynolds JG, McCoord AB and et al. Respiratory and electrolyte effects induced by estrogen and progesterone. *Fertil Steril* 1953;4:300-16.
44. Machida H. Influence of progesterone on arterial blood and CSF acid-base balance in women. *J Appl Physiol* 1981;51(6):1433-6.
45. Zwillich CW, Natalino MR, Sutton FD and Weil JV. Effects of progesterone on chemosensitivity in normal men. *J Lab Clin Med* 1978;92(2):262-9.
46. Skatrud JB, Dempsey JA and Kaiser DG. Ventilatory response to medroxyprogesterone acetate in normal subjects: time course and mechanism. *J Appl Physiol* 1978;44(6):393-44.
47. Lustik SJ, Chhibber AK and Kolano JW, et al. The hyperventilation of cirrhosis: progesterone and estradiol effects. *Hepatology* 1997;25(1):55-8.
48. Block AJ, Boysen PG, Wynne JW and Hunt LA. Sleep apnea, hypopnea and oxygen desaturation in normal subjects. A strong male predominance. *N Engl J Med* 1979;300(10):513-7.
49. Leech JA, Onal E, Dulberg C and Lopata MA. A comparison of men and women with occlusive sleep apnea syndrome. *Chest* 1988;94(5):983-8.
50. Block AJ, Wynne JW and Boysen PG. Sleep-disordered breathing and nocturnal oxygen desaturation in postmenopausal women. *Am J Med* 1980;69(1):75-9.
51. Bixler EO, Vgontzas AN and Lin HM, et al. Prevalence of sleep-disordered breathing in women: effects of gender. *Am J Respir Crit Care Med* 2001;163(3 Pt 1):608-13.
52. Hensley MJ, Saunders NA and Strohl KP. Medroxyprogesterone treatment of obstructive sleep apnea. *Sleep* 1980;3(3-4):441-6.
53. Orr WC, Imes NK and Martin RJ. Progesterone therapy in obese patients with sleep apnea. *Arch Intern Med* 1979;139(1):109-11.
54. Saaresranta T, Polo-Kantola P, Rauhala E and Polo O. Medroxyprogesterone in postmenopausal females with partial upper airway obstruction during sleep. *Eur Respir J* 2001;18(6):989-95.
55. Leavitt WW, Blaha GC. An estrogen-stimulated, progesterone-binding system in the hamster uterus and vagina. *Steroids* 1972;19(2):263-74.

56. Pickett CK, Regensteiner JG, Woodard WD, Hagerman DD, Weil JV and Moore LG. Progesterin and estrogen reduce sleep-disordered breathing in postmenopausal women. *J Appl Physiol* 1989;66(4):1656-61.
57. Cistulli PA, Barnes DJ, Grunstein RR and Sullivan CE. Effect of short-term hormone replacement in the treatment of obstructive sleep apnoea in postmenopausal women. *Thorax* 1994;49(7):699-702.
58. Keefe DL, Watson R and Naftolin F. Hormone replacement therapy may alleviate sleep apnea in menopausal women: a pilot study. *Menopause* 1999;6(3):196-200.
59. Hosenpud JD, Hart MV, Morton MJ, Hohimer AR and Resko JA. Progesterone-induced hyperventilation in the guinea pig. *Respir Physiol* 1983;52(2):259-64.
60. Keith IM, Bisgard GE, Manohar M, Klein J and Bullard VA. Respiratory effects of pregnancy and progesterone in Jersey cows. *Respir Physiol* 1982;50(3):351-8.
61. Nemets NM. Analysis of the characteristics and significance of change in internal environment of the body during pregnancy. *Fed Proc Transl Suppl* 1964;23:401-3.
62. Hoversland AS, Metcalfe J and Parer JT. Adjustments in maternal blood gases, acid-base balance, and oxygen consumption in the pregnant pygmy goat. *Biol Reprod* 1974;10(5):589-95.
63. Smith CA, Kellogg RH. Ventilatory response of rabbits and goats to chronic progesterone administration. *Respir Physiol* 1980;39(3):383-91.
64. Brodeur P, Mockus M, McCullough R and Moore LG. Progesterone receptors and ventilatory stimulation by progestin. *J Appl Physiol* 1986;60(2):590-5.
65. Tatsumi K, Mikami M, Kuriyama T and Fukuda Y. Respiratory stimulation by female hormones in awake male rats. *J Appl Physiol* 1991;71(1):37-42.
66. Bayliss DA, Cidlowski JA and Millhorn DE. The stimulation of respiration by progesterone in ovariectomized cat is mediated by an estrogen-dependent hypothalamic mechanism requiring gene expression. *Endocrinology* 1990;126(1):519-27.
67. Dutton K, Blanksby BA and Morton AR. CO₂ sensitivity changes during the menstrual cycle. *J Appl Physiol* 1989;67(2):517-22.
68. Regensteiner JG, Woodard WD and Hagerman DD, et al. Combined effects of female hormones and metabolic rate on ventilatory drives in women. *J Appl Physiol* 1989;66(2):808-13.
69. Jensen D, Wolfe LA, Slatkovska L, Webb KA, Davies GA and O'Donnell DE. Effects of human pregnancy on the ventilatory chemoreflex response to carbon dioxide. *Am J Physiol Regul Integr Comp Physiol* 2005;288(5):R1369-75.

70. Behan M, Thomas CF. Sex hormone receptors are expressed in identified respiratory motoneurons in male and female rats. *Neuroscience* 2005;130(3):725-34.
71. Mortola JP, Frappell PB. On the barometric method for measurements of ventilation, and its use in small animals. *Can J Physiol Pharmacol* 1998;76(10-11):937-44.
72. Phillipson EA, Murphy E and Kozar LF. Regulation of respiration in sleeping dogs. *J Appl Physiol* 1976;40(5):688-93.
73. Douglas NJ, White DP, Weil JV, Pickett CK and Zwillich CW. Hypercapnic ventilatory response in sleeping adults. *Am Rev Respir Dis* 1982;126(5):758-62.
74. Ryan KD, Schwartz NB. Changes in serum hormone levels associated with male-induced ovulation in group-housed adult female mice. *Endocrinology* 1980;106(3):959-66.
75. Michael SD. Plasma prolactin and progesterone during the estrous cycle in the mouse. *Proc Soc Exp Biol Med* 1976;153(2):254-7.
76. Fernandez SM, Frick KM. Chronic oral estrogen affects memory and neurochemistry in middle-aged female mice. *Behav Neurosci* 2004;118(6):1340-51.
77. Gordon MN, Osterburg HH, May PC and Finch CE. Effective oral administration of 17 beta-estradiol to female C57BL/6J mice through the drinking water. *Biol Reprod* 1986;35(5):1088-95.
78. Nillius SJ, Johansson ED. Plasma levels of progesterone after vaginal, rectal, or intramuscular administration of progesterone. *Am J Obstet Gynecol* 1971;110(4):470-7.
79. McDonnell AC, Van Kirk EA, Isaak DD and Murdoch WJ. Effects of progesterone on ovarian tumorigenesis in xenografted mice. *Cancer Lett* 2005;221(1):49-53.
80. Drorbaugh JE, Fenn WO. A barometric method for measuring ventilation in newborn infants. *Pediatrics* 1955;16(1):81-7.
81. Ding YQ, Marklund U and Yuan W, et al. Lmx1b is essential for the development of serotonergic neurons. *Nat Neurosci* 2003;6(9):933-8.
82. Richerson GB. Regulation of Respiration. In: Boron W, Boulpaep EL, ed. *Medical Physiology*. 1st ed. W.B. Saunders, 2002:713.
83. O'Regan RG, Majcherczyk S. Role of peripheral chemoreceptors and central chemosensitivity in the regulation of respiration and circulation. *J Exp Biol* 1982;100:23-40.
84. DeKosky ST, Marek K. Looking backward to move forward: early detection of neurodegenerative disorders. *Science* 2003;302(5646):830-4.

85. Oyamada Y, Ballantyne D, Muckenhoff K and Scheid P. Respiration-modulated membrane potential and chemosensitivity of locus coeruleus neurones in the in vitro brainstem-spinal cord of the neonatal rat. *J Physiol* 1998;513 (Pt 2)(Pt 2):381-98.
86. Pineda J, Aghajanian GK. Carbon dioxide regulates the tonic activity of locus coeruleus neurons by modulating a proton- and polyamine-sensitive inward rectifier potassium current. *Neuroscience* 1997;77(3):723-43.
87. Filosa JA, Dean JB and Putnam RW. Role of intracellular and extracellular pH in the chemosensitive response of rat locus coeruleus neurones. *J Physiol* 2002;541(Pt 2):493-509.
88. Nattie EE, Fung ML, Li A and St John WM. Responses of respiratory modulated and tonic units in the retrotrapezoid nucleus to CO₂. *Respir Physiol* 1993;94(1):35-50.
89. Teppema LJ, Berkenbosch A, Veening JG and Olievier CN. Hypercapnia induces c-fos expression in neurons of retrotrapezoid nucleus in cats. *Brain Res* 1994;635(1-2):353-6.
90. Dean JB, Bayliss DA, Erickson JT, Lawing WL and Millhorn DE. Depolarization and stimulation of neurons in nucleus tractus solitarii by carbon dioxide does not require chemical synaptic input. *Neuroscience* 1990;36(1):207-16.
91. Dean JB, Lawing WL and Millhorn DE. CO₂ decreases membrane conductance and depolarizes neurons in the nucleus tractus solitarii. *Exp Brain Res* 1989;76(3):656-61.
92. Shastry BS. More to learn from gene knockouts. *Mol Cell Biochem* 1994;136(2):171-82.
93. Morozov A, Kellendonk C, Simpson E and Tronche F. Using conditional mutagenesis to study the brain. *Biol Psychiatry* 2003;54(11):1125-33.
94. Rubinow DR, Schmidt PJ and Roca CA. Estrogen-serotonin interactions: implications for affective regulation. *Biol Psychiatry* 1998;44(9):839-50.
95. Bethea CL, Lu NZ, Gundlach C and Streicher JM. Diverse actions of ovarian steroids in the serotonin neural system. *Front Neuroendocrinol* 2002;23(1):41-100.
96. Enmark E, Peltö-Huikko M and Grandien K, et al. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* 1997;82(12):4258-65.
97. Giangrande PH, McDonnell DP. The A and B isoforms of the human progesterone receptor: two functionally different transcription factors encoded by a single gene. *Recent Prog Horm Res* 1999;54:291,313; discussion 313-4.
98. Vanderhorst VG, Gustafsson JA and Ulfhake B. Estrogen receptor-alpha and -beta immunoreactive neurons in the brainstem and spinal cord of male and female mice:

relationships to monoaminergic, cholinergic, and spinal projection systems. *J Comp Neurol* 2005;488(2):152-79.

99. Nomura M, Akama KT and Alves SE, et al. Differential distribution of estrogen receptor (ER)-alpha and ER-beta in the midbrain raphe nuclei and periaqueductal gray in male mouse: Predominant role of ER-beta in midbrain serotonergic systems. *Neuroscience* 2005;130(2):445-56.

100. Alves SE, McEwen BS, Hayashi S, Korach KS, Pfaff DW and Ogawa S. Estrogen-regulated progestin receptors are found in the midbrain raphe but not hippocampus of estrogen receptor alpha (ER alpha) gene-disrupted mice. *J Comp Neurol* 2000;427(2):185-95.

101. Bethea CL. Regulation of progestin receptors in raphe neurons of steroid-treated monkeys. *Neuroendocrinology* 1994;60(1):50-61.

102. Lu NZ, Shlaes TA, Gundlah C, Dziennis SE, Lyle RE and Bethea CL. Ovarian steroid action on tryptophan hydroxylase protein and serotonin compared to localization of ovarian steroid receptors in midbrain of guinea pigs. *Endocrine* 1999;11(3):257-67.

103. Bethea CL, Mirkes SJ, Shively CA and Adams MR. Steroid regulation of tryptophan hydroxylase protein in the dorsal raphe of macaques. *Biol Psychiatry* 2000;47(6):562-76.

104. Gundlah C, Alves SE, Clark JA, Pai LY, Schaeffer JM and Rohrer SP. Estrogen receptor-beta regulates tryptophan hydroxylase-1 expression in the murine midbrain raphe. *Biol Psychiatry* 2005;57(8):938-42.

105. McQueen JK, Wilson H and Fink G. Estradiol-17 beta increases serotonin transporter (SERT) mRNA levels and the density of SERT-binding sites in female rat brain. *Brain Res Mol Brain Res* 1997;45(1):13-23.

106. Pecins-Thompson M, Brown NA and Bethea CL. Regulation of serotonin re-uptake transporter mRNA expression by ovarian steroids in rhesus macaques. *Brain Res Mol Brain Res* 1998;53(1-2):120-9.

107. Bertrand PP, Parnavitane UT, Chavez C, Gogos A, Jones M and van den Buuse M. The effect of low estrogen state on serotonin transporter function in mouse hippocampus: a behavioral and electrochemical study. *Brain Res* 2005;1064(1-2):10-20.

108. Pecins-Thompson M, Bethea CL. Ovarian steroid regulation of serotonin-1A autoreceptor messenger RNA expression in the dorsal raphe of rhesus macaques. *Neuroscience* 1999;89(1):267-77.

109. Gundlah C, Lu NZ and Bethea CL. Ovarian steroid regulation of monoamine oxidase-A and -B mRNAs in the macaque dorsal raphe and hypothalamic nuclei. *Psychopharmacology (Berl)* 2002;160(3):271-82.

110. Bethea CL, Pecins-Thompson M, Schutzer WE, Gundlah C and Lu ZN. Ovarian steroids and serotonin neural function. *Mol Neurobiol* 1998;18(2):87-123.
111. Klink R, Robichaud M and Debonnel G. Gender and gonadal status modulation of dorsal raphe nucleus serotonergic neurons. Part I: effects of gender and pregnancy. *Neuropharmacology* 2002;43(7):1119-28.
112. Klink R, Robichaud M and Debonnel G. Gender and gonadal status modulation of dorsal raphe nucleus serotonergic neurons. Part II. Regulatory mechanisms. *Neuropharmacology* 2002;43(7):1129-38.
113. Robichaud M, Debonnel G. Modulation of the firing activity of female dorsal raphe nucleus serotonergic neurons by neuroactive steroids. *J Endocrinol* 2004;182(1):11-21.
114. Robichaud M, Debonnel G. Oestrogen and testosterone modulate the firing activity of dorsal raphe nucleus serotonergic neurones in both male and female rats. *J Neuroendocrinol* 2005;17(3):179-85.
115. Lu H, Nishi M, Matsuda K and Kawata M. Estrogen reduces the neurite growth of serotonergic cells expressing estrogen receptors. *Neurosci Res* 2004;50(1):23-8.
116. McEwen BS, Alves SE. Estrogen actions in the central nervous system. *Endocr Rev* 1999;20(3):279-307.



3 9002 07925 7110

HARVEY CUSHING/JOHN HAY WHITNEY MEDICAL LIBRARY

MANUSCRIPT THESES

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by
has been used by the following person, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS

DATE

